# Isolation and killing of candidate chronic myeloid leukemia stem cells by antibody targeting of IL-1 receptor accessory protein

Marcus Järås[a], Petra Johnels[a], Nils Hansen[a], Helena Ågerstam[a], Panagiotis Tsapogas[a], Marianne Rissler[a], Carin Lassen[a], Tor Olofsson[b], Ole Weis Bjerrum[c], Johan Richter[d], and Thoas Fioretos[a,1]

[a]Department of Clinical Genetics, University and Regional Laboratories, Skåne University Hospital, Lund University, 22185 Lund, Sweden; Departments of [b]Hematology and [d]Molecular Medicine and Gene Therapy, Lund University, 22184 Lund, Sweden; and [c]Department of Hematology, Rigshospitalet, University of Copenhagen, 2100 Copenhagen, Denmark

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**Chronic myeloid leukemia (CML) is genetically characterized by the Philadelphia (Ph) chromosome, formed through a reciprocal translocation between chromosomes 9 and 22 and giving rise to the constitutively active tyrosine kinase P210 BCR/ABL1. Therapeutic strategies aiming for a cure of CML will require full eradication of Ph chromosome-positive ($Ph^+$) CML stem cells. Here we used gene-expression profiling to identify IL-1 receptor accessory protein (IL1RAP) as up-regulated in CML $CD34^+$ cells and also in cord blood $CD34^+$ cells as a consequence of retroviral *BCR/ABL1* expression. To test whether IL1RAP expression distinguishes normal ($Ph^-$) and leukemic ($Ph^+$) cells within the CML $CD34^+CD38^-$ cell compartment, we established a unique protocol for conducting FISH on small numbers of sorted cells. By using this method, we sorted cells directly into drops on slides to investigate their Ph-chromosome status. Interestingly, we found that the CML $CD34^+CD38^-IL1RAP^+$ cells were $Ph^+$, whereas CML $CD34^+CD38^-IL1RAP^-$ cells were almost exclusively $Ph^-$. By performing long-term culture-initiating cell assays on the two cell populations, we found that $Ph^+$ and $Ph^-$ candidate CML stem cells could be prospectively separated. In addition, by generating an anti-IL1RAP antibody, we provide proof of concept that IL1RAP can be used as a target on CML $CD34^+CD38^-$ cells to induce antibody-dependent cell-mediated cytotoxicity. This study thus identifies IL1RAP as a unique cell surface biomarker distinguishing $Ph^+$ from $Ph^-$ candidate CML stem cells and opens up a previously unexplored avenue for therapy of CML.**

antibody-dependent cell-mediated cytotoxicity | cancer | biomarker | therapeutic antibody

Chronic myeloid leukemia (CML) was the first human neoplasm to be associated with a recurrent genetic aberration, the Philadelphia (Ph) chromosome, formed through a reciprocal translocation between chromosomes 9 and 22 and giving rise to the constitutively active tyrosine kinase P210 BCR/ABL1 (1–4). In CML, the Ph chromosome is believed to originate in a hematopoietic stem cell (HSC) as it clonally can be found both in myeloid and lymphoid cells (5). CML consists of heterogeneous cell types at various maturation stages that are maintained by a small number of cells, termed CML stem cells, sharing with normal HSCs the capacity to self-renew (6). The CML stem cells are at least partially resistant to current treatments with tyrosine kinase inhibitors (TKIs) (7, 8) that, despite clinical success, show a suppressive rather than curative effect in this disease. Thus, to achieve a cure of CML, a desirable strategy is to efficiently target the CML stem cells. Achieving this goal would involve identifying a target on CML stem cells that could provide a novel means of eradicating them. Encouraging reports in this direction have been described in acute myeloid leukemia models, in which antibodies targeting CD123, CXCR4, CD44, or CD47 on acute myeloid leukemia stem cells show antileukemic effects (9–12).

Intriguingly, despite the fact that CML is one of the most studied neoplasms of all time and considered a prototypic cancer stem-cell disorder, no cell surface biomarker has so far been identified in this disorder that would allow prospective separation of CML stem cells from normal HSCs, both of which reside in the rare $CD34^+CD38^-$ cell population (13, 14). Identification of such a marker would not only be instrumental in characterizing CML stem cells but could also be used for the development of novel treatments and tracking therapeutic effects on primitive CML cells during treatment. To identify a cell surface biomarker for CML stem cells, we performed gene-expression profiling and identified IL-1 receptor accessory protein (IL1RAP) as the top candidate. With the development of an assay for detecting *BCR/ABL1* in low numbers of sorted cells and through long-term culturing-initiating cell (LTC-IC) assays, we further show that IL1RAP is a cell surface biomarker for putative CML stem cells. This finding is unique in allowing the prospective separation of such cells from normal HSCs. Finally, we generated an IL1RAP-targeting antibody that killed CML $CD34^+CD38^-$ cells, but not corresponding normal cells, through antibody-dependent cell-mediated cytotoxicity (ADCC), demonstrating a unique concept for the possible eradication of CML stem cells.

## Results

**Global Gene Expression Analysis Identifies IL1RAP as Up-Regulated in CML $CD34^+$ Cells.** Much effort has been put into investigations aimed at identifying a cell-surface biomarker for $Ph^+$ CML stem cells, as reviewed by Jiang et al. (15). However, so far, no cell-surface marker has been identified that would allow prospective separation of CML stem cells from normal HSCs. To search for up-regulated genes encoding cell-surface proteins on primitive CML cells, we performed global transcriptional profiling of $CD34^+$ cells from 10 chronic-phase CML patients and six healthy donors. Genes identified as up-regulated in CML were matched to the Gene Ontology (GO) category "integral to plasma membrane" (see *Materials and Methods* for details). In total, 13 up-regulated genes in CML $CD34^+$ cells matched to the selected GO category (Fig. 1*A*). To identify up-regulated genes more directly linked to P210 *BCR/ABL1* expression, we performed gene-expression analysis of cord blood $CD34^+$ cells following retroviral P210 *BCR/ABL1* expression in parallel. This analysis resulted in 23 up-regulated genes matching to the same GO category gene list (Fig. 1*B*). Interestingly, one gene, *IL1RAP*, was strongly up-regulated in both CML and in CB $CD34^+$ cells as a consequence of P210 *BCR/ABL1* expression. The occur-

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Conflict of interest statement: M.J and T.F are the inventors of patent applications to cover IL1RAP for diagnostic and therapeutic applications in leukemia. M.J. and T.F. have equity ownership in a company (Cantargia AB, Ideon Research Park, Lund, Sweden) formed together with Lund University Bioscience AB. Cantargia AB is the owner of the patent applications mentioned above.

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[1]To whom correspondence should be addressed. E-mail: thoas.fioretos@med.lu.se.

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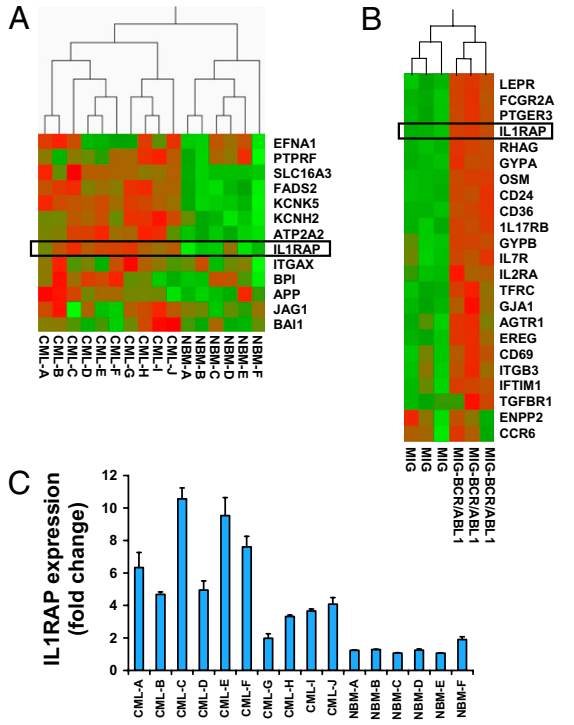


**Fig. 1.** Gene expression analysis identifies IL1RAP as up-regulated in CML CD34$^+$ cells and following retroviral P210 *BCR/ABL1* expression in CB CD34$^+$ cells. Global gene-expression analyses were performed on CD34$^+$ cells obtained at diagnosis from chronic-phase CML patients and on CB CD34$^+$ cells, 2 d after retroviral P210 *BCR/ABL1* transduction. Heatmaps showing up-regulated genes (red) and down-regulated genes (green) matching to the GO category "integral to plasma membrane" are displayed for primary CD34$^+$ cells obtained from normal bone marrows (NBM) ($n$ = 6) and CML (Ph$^+$) patients ($n$ = 10) (*A*) and for retroviral P210 *BCR/ABL1*-expressing cells in comparison with empty vector MIG control-expressing cells (*B*). *IL1RAP*, marked in the figure, is the only highly up-regulated gene in common between the two gene lists. The up-regulation of the *IL1RAP* transcript was confirmed by real-time PCR using 18S as endogenous control (*C*). *IL1RAP* expression is presented as fold change in relation to NBM-C.

rence of *IL1RAP* on both gene lists suggests that its up-regulation in primitive CML cells is closely coupled to P210 *BCR/ABL1* expression and identified IL1RAP as a strong candidate for being a unique leukemia-associated antigen on primitive CML cells. The finding of increased *IL1RAP* expression is in accordance with previous findings reporting transcriptional profiling of primitive CML cells (16, 17). The up-regulation of the *IL1RAP* transcript in CML CD34$^+$ cells was confirmed by real-time PCR (Fig.1*C*).

**IL1RAP Is Induced as a Consequence of Retroviral P210 *BCR/ABL1* Expression and Is Also Present on a Population of CD34$^+$CD38$^-$ Cells from CML Patients.** IL-1-induced IL-1 receptor-type 1 (IL-1R1) activation has previously been shown to stimulate colony growth of IFN-sensitive CML cells (18); however, its coreceptor IL1RAP has, to our knowledge, not previously been directly associated with *BCR/ABL1* and CML. Because P210 *BCR/ABL1* is present in CML cells as a hallmark of the disease, ideally a reliable cell-surface biomarker in this disorder should be directly coupled to the presence and expression of P210 *BCR/ABL1*. In agreement with the microarray gene-expression data, we confirmed by using a goat anti-human IL1RAP antibody and FACS analysis that the IL1RAP protein becomes up-regulated on the cell surface of CB CD34$^+$ cells following retroviral P210 *BCR/ABL1* expression (Fig. 2*A*). Interestingly, a BCR/ABL1 kinase inactive mutant was unable to cause IL1RAP up-regulation and imatinib treatment of the CML cell line KU812 caused down-regulation of IL1RAP (Fig. 2 *A* and *B*). These findings suggest that the kinase activity of P210 *BCR/ABL1* is important in regulating IL1RAP expression, either directly or through an indirect effect.

Next, we investigated the cell-surface IL1RAP expression on CML CD34$^+$CD38$^+$ progenitor cells from five CML patients. In this subpopulation of cells, up-regulation of IL1RAP was observed compared with low IL1RAP expression in corresponding normal bone-marrow cells (Fig. 3*A* and Fig. S1). We then turned to the more immature CD34$^+$CD38$^-$ cell compartment of normal cells containing the HSCs. In agreement with the results of a previous study of normal primitive hematopoietic cells, this population displayed low or absent IL1RAP expression (Fig. 3*B*) (19). Strikingly, the CD34$^+$CD38$^-$ cells from CML patients, harboring both Ph$^+$ and Ph$^-$ CML stem cells, could be divided into two populations: one exhibiting low or absent IL1RAP expression and the other having higher IL1RAP expression (Fig. 3*B*). The IL1RAP$^+$cell fraction constituted between 75% and 95% of the CML CD34$^+$CD38$^-$ cells ($n$ = 5), corresponding to about 1 in 1,300 mononuclear cells; the more rare CD34$^+$CD38$^-$IL1RAP$^-$ cells corresponded to about 1 in 11,000 mononuclear cells.

**Flow-Drop-FISH Shows That IL1RAP Expression Separates Normal and Leukemic Cells Within CML CD34$^+$CD38$^-$ Cells.** To test whether IL1RAP expression distinguishes Ph$^-$ and Ph$^+$ cells within the CD34$^+$CD38$^-$ cell compartment in CML, we applied FISH to detect the *BCR/ABL1* rearrangement in cells sorted according to the gates in Fig. 3*B*. Because the CML CD34$^+$CD38$^-$IL1RAP$^-$ cell subset is a rare cell population, we encountered major challenges in performing FISH on flow-sorted cells using standard protocols (20). Hence, to test whether IL1RAP expression distinguishes normal (Ph$^-$) and leukemic (Ph$^+$) cells within the CML CD34$^+$CD38$^-$ cell compartment, we established a unique protocol for conducting FISH on small numbers of sorted cells (*Materials and Methods*). The first steps in this protocol are based partly on a method for sorting cells into drops on slides followed by single-cell immunostaining (21). With this unique protocol involving cell sorting directly into drops on slides followed by FISH, hereafter referred to as Flow-drop-FISH, we could sort as few as 30 cells into a drop, from which 15 nuclei were successfully scored by FISH (Fig. 4). Interestingly, by using Flow-drop-FISH, we found that the CML CD34$^+$CD38$^-$IL1RAP$^+$ cells were *BCR/ABL1*$^+$ (99.9 ± 0.2% Ph$^+$, $n$ = 5), whereas CML CD34$^+$CD38$^-$IL1RAP$^-$ cells were almost exclusively *BCR/ABL1*$^-$ (97.1 ± 3.4% Ph$^-$, $n$ = 5) (Fig. 4). These data show that IL1RAP expression separates leukemic and normal cells within the CD34$^+$CD38$^-$ cell compartment of CML patients at diagnosis.

**IL1RAP Expression Distinguishes Ph$^+$ from Ph$^-$ Candidate CML Stem Cells.** Chronic-phase CML stem cells have unexpectedly been demonstrated to have reduced self-renewal ability (22), and because these cells generally show poor or no engraftment in immunodeficient mice, the LTC-IC assay has become a widely used surrogate assay for detecting candidate CML stem cells (15). To test whether the CML CD34$^+$CD38$^-$IL1RAP$^+$ and CD34$^+$CD38$^-$IL1RAP$^-$ subpopulations of cells separate Ph$^+$ and Ph$^-$ candidate CML stem cells, we investigated the two cell populations using LTC-IC assays. Following seeding of CML CD34$^+$CD38$^-$IL1RAP$^-$ cells on stroma cells, we observed on average a 3.6-fold higher number of long-term culture colony-forming cells (LTC-CFCs) compared with the numbers of colonies derived from CD34$^+$CD38$^-$IL1RAP$^+$ cells (Fig. 5*A*). For corresponding normal control cells, LTC-CFCs were found at on average 50-fold higher numbers from CD34$^+$CD38$^-$IL1RAP$^-$ cells than from CD34$^+$CD38$^-$IL1RAP$^{+/low}$ cells (Fig. 5*A*), suggesting that normal HSCs have a CD34$^+$CD38$^-$IL1RAP$^-$ immunophenotype. Of importance, although a higher frequency of LTC-ICs were found among CML CD34$^+$CD38$^-$IL1RAP$^-$ cells than within CML CD34$^+$CD38$^-$IL1RAP$^+$ cells, FISH on cells from CML LTC colonies revealed an almost complete discrimination between Ph$^-$ and Ph$^+$ cells in the two groups (Fig. 5*B*). CML LTC colonies derived from CD34$^+$CD38$^-$IL1RAP$^-$ cells were almost exclusively Ph$^-$, whereas colonies originating from CD34$^+$CD38$^-$IL1RAP$^+$ cells were almost exclusively Ph$^+$. It should be noted that Ph$^-$ LTC-ICs are found at higher frequencies in the circulation of untreated CML patients than in healthy controls; thus, the finding of these cells in the peripheral blood of CML patients is expected (23). Collectively,

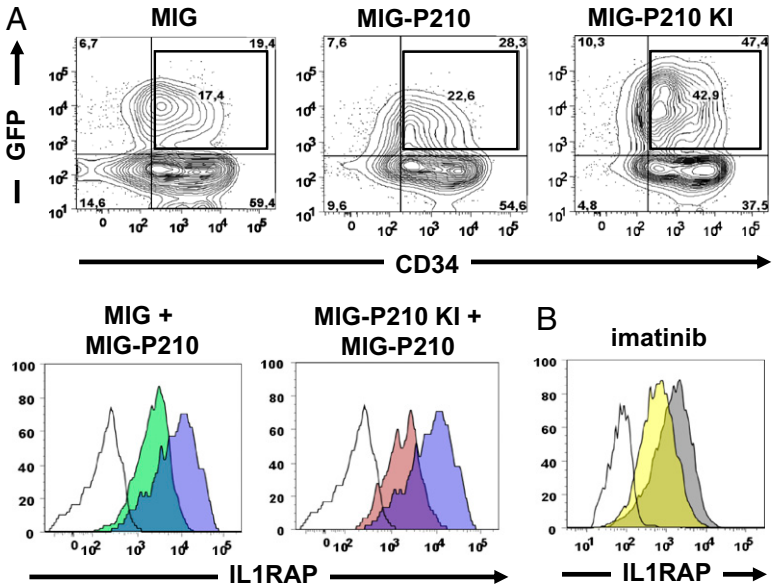


**Fig. 2.** The kinase activity of P210 *BCR/ABL1* induces up-regulation of IL1RAP on the cell surface. Flow cytometric analysis confirmed that IL1RAP expression is induced upon retroviral P210 *BCR/ABL1* expression of cord blood CD34$^+$ cells, 3 d after transduction (*A*). CD34$^+$GFP$^+$ cells were gated according to the gates in the contour plots. The histogram shows the expression of IL1RAP for negative-control staining (white), MIG control (green), MIG-P210 (blue), and MIG-P210 kinase inactive (KI) (light red). KU812 cells were also treated with 5 μM imatinib and IL1RAP expression was assessed 2 d later in live cells (7-AAD$^-$) (*B*). The histogram shows the expression of IL1RAP for imatinib-treated cells (yellow) and nonimatinib-treated cells (gray). One representative experiment out of three is shown. MIG; MSCV-IRES-GFP, murine stem cell virus-internal ribosome entry site-green fluorescent protein.

these data show that IL1RAP is a unique cell-surface biomarker that can be used to separate Ph$^+$ from Ph$^-$ CML LTC-IC.

**Antibody Targeting of IL1RAP on CML CD34$^+$CD38$^-$ Cells Directs Natural Killer Cells to ADCC.** Several therapeutic antibodies, such as Rituximab directed against CD20, are believed to at least partially exert their therapeutic effect through ADCC (24). To test whether ADCC could be achieved using IL1RAP as a target, we generated a polyclonal rabbit anti-human IL1RAP antibody, designated KMT-1; we selected rabbit antibodies because human immune-system cells efficiently recognize the Fc domains of such antibodies, in contrast to goat and mouse antibodies (25). The ability of KMT-1 and a control IgG antibody to induce cell death was investigated using human natural killer (NK) cells as effector cells.

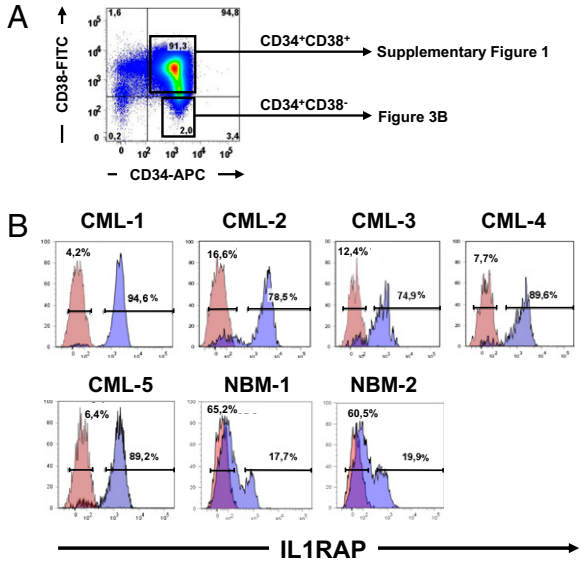


**Fig. 3.** IL1RAP is up-regulated on the cell surface of CML CD34$^+$CD38$^-$ cells. FACS analysis of CD34$^+$ cells from five CML patients in chronic-phase (CML1-5) and from two NBM samples (NBM1, -2). (*A*) FACS dot-plot showing gating for CD34$^+$CD38$^+$ or CD34$^+$CD38$^-$ cells in a representative CML patient. (*B*) Histogram showing IL1RAP expression within CD34$^+$CD38$^-$ cells. Light red represents control-stained samples and blue represents IL1RAP-stained samples. The sorting gates for CD34$^+$CD38$^-$IL1RAP$^-$ and CD34$^+$CD38$^-$IL1RAP$^+$ cells are outlined in the histograms. IL1RAP is up-regulated in CML CD34$^+$CD38$^-$ cells compared with their normal counterparts.

In the IL1RAP$^-$ Ph$^-$ leukemia cell line KG-1, lacking IL1RAP expression (Fig. 6*A* and Fig. S2 *A* and *B*), only a low level of ADCC was observed, even at high KMT-1 concentrations (Fig. 6*B*). In contrast, in the Ph$^+$ CML cell line KU812, expressing IL1RAP (Fig. 6*A* and Fig. S2 *A* and *B*), relatively high ADCC was observed in the presence of KMT-1 (Fig. 6*B*), demonstrating that KMT-1 has the potential to induce ADCC by binding IL1RAP on leukemic target cells. Moreover, blocking IL1RAP using the goat anti-IL1RAP antibody before the addition of KMT-1 caused a significant reduction in the ADCC effect, supporting the theory that KMT-1 induces ADCC in an IL1RAP-dependent manner (Fig. S3). In addition, we assessed the IL1RAP expression in six more CML cell lines that all stained positive for IL1RAP (Fig. S4). On primary cells from CML patients and normal controls, KMT-1 showed a slightly weaker but similar staining pattern as that obtained with the previously used polyclonal goat anti-human IL1RAP antibody (Fig. S5). Immature cells from CML-1, CML-3, and CML-4 were tested in ADCC assays in parallel to cells from healthy control samples. In CML CD34$^+$ cells, the binding of KMT-1 mediated ADCC at higher levels than in normal CD34$^+$ control cells, correlating with the expression level of IL1RAP, in particular at lower antibody concentrations (Fig. 6*C*). More strikingly, among the stem cell-enriched CD34$^+$CD38$^-$ cells, KMT-1 did not induce ADCC of normal CD34$^+$CD38$^-$ cells, whereas a clear dose-dependent ADCC effect was observed in CML CD34$^+$CD38$^-$ cells (Fig. 6*D*).

To address the selectivity of IL1RAP-targeting antibodies, we also characterized the IL1RAP expression on various cell populations of normal hematopoiesis. With the exception of monocytes showing a substantial IL1RAP expression, IL1RAP expression was relatively low or absent on most types of normal progenitor and mature cell populations (Fig. S6).

## Discussion

In the present study, we have identified IL1RAP as a unique cell surface biomarker that distinguishes candidate CML stem cells from normal HSCs and used this knowledge to induce an antibody-dependent cell killing of CML CD34$^+$CD38$^-$ cells. This finding opens up a previously unexplored possible strategy to treat CML by direct targeting of the CML stem cells, a concept that is distinct from the TKI therapies currently used, which preferentially are effective against cells downstream of the CML stem cells (8, 26).

The reason that CML stem cells are partially resistant to TKIs, such as imatinib (Gleevec, Novartis Inc.) is unclear, but factors that have been suggested to contribute to this resistance include quiescence, relatively high levels of *BCR/ABL1* expression, ac-

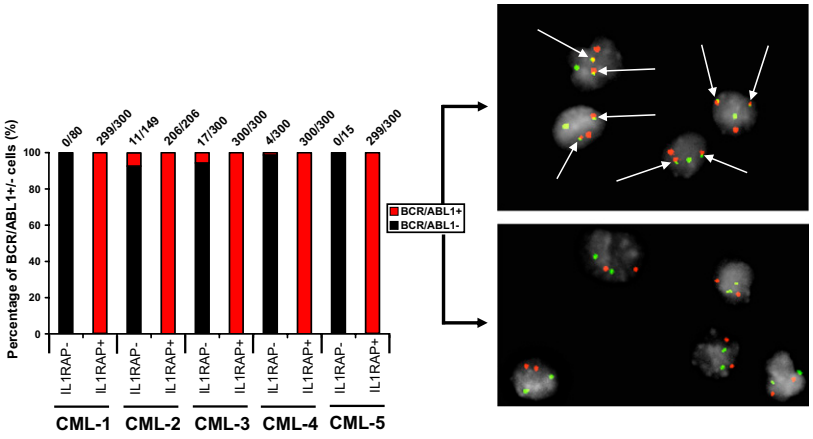


**Fig. 4.** IL1RAP expression distinguishes Ph$^+$ from Ph$^-$ CML cells within the CD34$^+$CD38$^-$ cell compartment. Flow-drop-FISH on CML CD34$^+$CD38$^-$IL1RAP$^-$ and CD34$^+$CD38$^-$IL1-RAP$^+$ cells from CML1-5 revealed an almost complete separation between Ph$^-$ and Ph$^+$ cells, respectively. (*Left*) Black bars represent *BCR/ABL1*$^-$ cells and red bars represent *BCR/ABL1*$^+$ cells. Outlined at the top of each bar is the number of Ph$^+$ cells of the total nuclei scored within individual samples. The IL1RAP status of sorted cells is shown beneath each bar. (*Right*) Pictures show BCR/ABL1$^+$ (one red, one green, and two fused signals) versus BCR/ABL1$^-$ (two green and two red signals) cells. White arrows points to fused signals.

quired mutations in *BCR/ABL1*, and combinatorial expression of specific membrane transporter proteins in these cells (7, 8, 27–29). Given these features, novel treatment approaches to ultimately eradicate the CML stem cells are highly desirable. One such strategy is an antibody-based therapy directly targeting CML stem cells, in which the antibody mode of action is independent of the known resistance mechanisms causing CML stem cells to be unresponsive to TKI treatment. The major limitations for such developments have so far been the lack of a cell surface receptor distinguishing Ph$^+$ from Ph$^-$ CML stem cells. By using global gene expression analyses of primary CML cells, we identified IL1RAP as a candidate target. IL1RAP is a member of the Toll-like receptor superfamily and is a well-known coreceptor of IL-1R1 (30). IL1RAP is thus crucial in mediating the effect of the proinflammatory cytokine IL-1, but it is also involved in mediating the signal of IL-33, a cytokine that activates T cells and mast cells through binding of its receptor ST2, which subsequently dimerizes with IL1RAP (31).

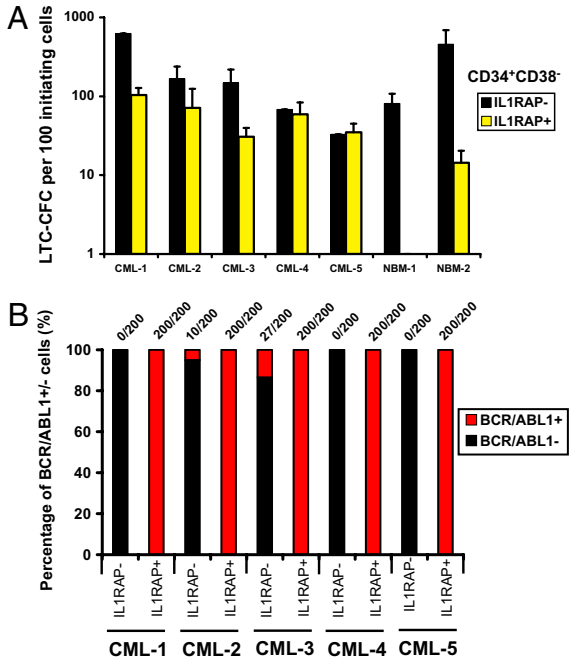


**Fig. 5.** IL1RAP expression distinguishes Ph$^+$ from Ph$^-$ CML LTC-IC. (*A*) Number of LTC-CFCs derived from CD34$^+$CD38$^-$IL1RAP$^-$ and CD34$^+$CD38$^-$IL1RAP$^+$ cells. Black bars represent IL1RAP$^-$ cells and yellow bars represent IL1RAP$^+$ cells. (*B*) Interphase FISH on LTC-CFC. Black bars represent BCR/ABL1$^-$ cells and red bars represent BCR/ABL1$^+$ cells. Outlined at the top of each bar is the number of Ph$^+$ cells of the total nuclei scored within individual samples. The IL1RAP status of sorted LTC-IC is shown beneath each bar. A near-complete discrimination of BCR/ABL1$^-$ and BCR/ABL1$^+$ cells was found among LTC colonies derived from CD34$^+$CD38$^-$IL1RAP$^-$ or CD34$^+$CD38$^-$IL1RAP$^+$ cells, respectively.

Although we did not address whether IL1RAP is functionally involved in leukemogenesis, we did find that IL1RAP becomes up-regulated on the cell surface of CB CD34$^+$ cells following retroviral expression of *BCR/ABL1*. This result suggests that IL1RAP is regulated by the well-known signaling cascades that BCR/ABL1 activates (4, 32).

By using Flow-drop-FISH and LTC-IC assays, we demonstrated that IL1RAP can be used for prospective separation of Ph$^+$ and Ph$^-$ candidate CML stem cells. Although it would be desirable to test the stem-cell properties of these two cell populations in immunodeficient mice, the low numbers of sorted CML cells acquired from the IL1RAP$^-$ and IL1RAP$^+$ CD34$^+$CD38$^-$ cell subpopulations, together with the general low efficiency in engrafting chronic phase CML cells in these mice (33), prevented us from successfully performing such experiments. Nevertheless, the near-complete separation of Ph$^+$ and Ph$^-$ CML cells based on IL1RAP expression within the CD34$^+$CD38$^-$ population and their separation in LTC-IC assays suggest that IL1RAP expression can be used to prospectively isolate Ph$^+$ from Ph$^-$ candidate CML stem cells. It cannot entirely be excluded that extremely rare Ph$^+$ CML stem cells might be contained within the CD34$^+$CD38$^-$IL1RAP$^-$ cell fraction; however, because on average 97.1 ± 3.4% of the CD34$^+$CD38$^-$IL1RAP$^-$ cells were Ph$^-$, we find this possibility less likely.

As demonstrated in this study, the ability to separate Ph$^+$ from Ph$^-$ candidate CML stem cells should also enable future detailed mechanistic studies of these two cell populations in CML. In addition, the Flow-drop-FISH protocol could serve as a useful method for characterizing genetic aberrations in small numbers of sorted cells, such as leukemic stem cells (34). Furthermore, in particular for CML in which leukemic cells can be traced by detection of *BCR/ABL1* using FISH, Flow-drop-FISH can be used to monitor residual immature leukemic cells during treatment.

Importantly, by generating an antibody that targets IL1RAP, we are unique in providing proof of concept that CML CD34$^+$CD38$^-$ cells can be targeted while preserving corresponding normal cells. Notably, we have not directly demonstrated that the ADCC effect exclusively targets the Ph$^+$ CML cells within the CD34$^+$CD38$^-$ cell subset. However, given that on average 86% of the cells within the CD34$^+$CD38$^-$ subpopulation in the patients tested were Ph$^+$, and the almost undetectable ADCC in CD34$^+$CD38$^-$ cells from normal controls, our data strongly support the idea that the IL1RAP-targeting antibodies indeed induce ADCC preferentially in Ph$^+$ CML CD34$^+$CD38$^-$ cells.

Therapeutic antibodies are commonly administered intravenously, and selectivity and specificity becomes a major concern for reducing toxicity (24). IL1RAP expression was present in monocytes, but was low or absent in most types of normal bone-marrow progenitor and mature cell types, suggesting that although monocytes should be monitored carefully, potential future therapeutic IL1RAP-targeting antibodies are expected to show low toxicity on normal hematopoietic cells. However, given the weak but present

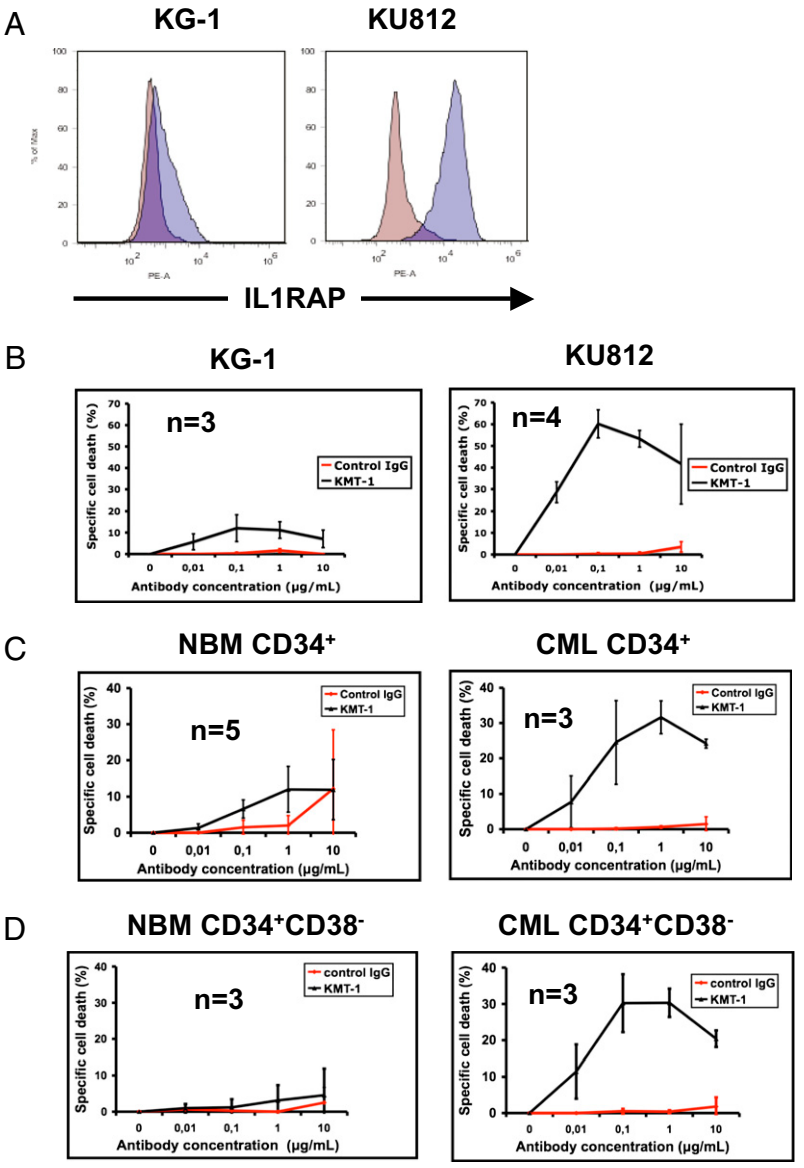


**Fig. 6.** Killing of CML CD34$^+$CD38$^-$ cells by antibody targeting of IL1RAP. (*A*) Histogram showing IL1RAP expression in the Ph$^-$ KG-1 and Ph$^+$ KU812 cell lines. Light red shows control-stained samples and blue shows KMT-1–stained samples. The leukemic cell line KG-1 is devoid of IL1RAP expression, whereas KU812 cells express IL1RAP. As a consequence, only a low level of antibody-induced cell death was observed in KG-1, but a dose-dependent ADCC effect was observed using KMT-1 on KU812 cells (*B*). In line with the level of IL1RAP expression, no obvious ADCC effect was seen using NBM CD34$^+$ (*C*) and CD34$^+$CD38$^-$ (*D*) cells, whereas KMT-1 induced a strong dose-dependent ADCC effect in both CML CD34$^+$ (*C*) and CML CD34$^+$CD38$^-$ cells (*D*). As a control for nonspecific ADCC effects, a rabbit IgG antibody was also used in the experiments. Each graph shows the average and SD of antibody-induced cell death from a minimum of three independent experiments.

IL1RAP expression in mature lymphocytes and the role of IL1RAP in IL-33–mediated activation of T lymphocytes and mast cells, these cell types should also be monitored carefully.

Finally, it is worth noting that because the antibody mode of action in ADCC is to direct immunological cells to target cell killing, the therapeutic mechanism here is independent of known mechanisms of TKI resistance in CML. Hence, we hypothesize that the concept of antibody-mediated killing of IL1RAP-expressing CML stem cells may also have the potential to eradicate such cells in patients, either alone or in combination with current regimens, ultimately leading to a cure for CML patients.

## Materials and Methods

**Collection of CML Patient Cells.** Blood samples from CML patients at diagnosis were obtained after informed consent and before treatment was initiated, according to a protocol approved by the regional, research ethics committee, Lund University. Samples were received both from the Department of Hematology at Skåne University Hospital, Sweden, and from Rigshospitalet, Copenhagen, Denmark. For CML patient characteristics, see Table S1. Mononuclear cells were separated using Lymphoprep (Axis-Shield PoC AS) according to the manufacturer's instructions, and CD34$^+$ cells were enriched using the CD34$^+$ cell isolation kit (Miltenyi Biotec), as previously described (35). This separation yielded a purity of CD34$^+$ cells above 95%. CD34$^+$ cells were split into two fractions; one fraction was washed in PBS, resuspended in TRIzol, and frozen at −80 °C, whereas the other fraction was frozen in liquid nitrogen. As reference samples, bone marrow samples from healthy volunteers were obtained after informed consent at the Skåne University Hospital, followed by CD34 cell enrichment as described above.

**P210 *BCR/ABL1* Expression in Cord Blood CD34$^+$ Cells.** Umbilical cord blood CD34$^+$ cells were enriched and transduced as previously described (36). See *SI Materials and Methods* for details.

**Microarray Analysis and Real-Time PCR.** Microarray analyses on patient samples were performed using oligonucleotide arrays from the Swegene DNA Microarray Resource Center at Lund University, Sweden. For details on the microarray analysis, see *SI Materials and Methods*. Real-time PCR analysis was performed using an ABI Prism 7500 analyzer (Applied Biosystems) and standard protocols. All samples were analyzed in triplicate. Primers and probes for IL1RAP were ordered from Applied Biosystems as assay-on-demand primers. The relative quantity was calculated based on the ΔΔCt method (37) and normalized to 18S rRNA.

**Flow Cytometric Analysis.** Flow cytometric analyses were performed in a FACSCanto flow cytometer, and flow cytometric cell sorting was done in a FACSAria cell sorter (both from Becton-Dickinson Immunocytometry Systems). See *SI Materials and Methods* for details on the flow cytometric analysis.

**Flow-Drop-FISH.** Glass slides were treated with 0.01% poly-L-lysine (Sigma-Aldrich) for 2 h and kept in a moist chamber, then washed once in water and dried on a hot plate at 37 °C. Subsequently, a hydrophobic pen (Daido Sangyo Co.) was used to draw circles with a 96-well tissue culture plate as template. Before cell sorting, 25 μL PBS was applied to the rings to form drops. During cell sorting, 30 to 3,000 cells were sorted in parallel directly into two drops. To allow attachment of the cells to the surface and to avoid drying of the drops, slides were maintained in a moist chamber on ice for 30 min before cells were fixed in methanol:acetic acid (3:1) for 10 min. Subsequently, slides were incubated in a 70 °C oven overnight, followed by FISH analysis, as previously described (38). Dual-color probes for *BCR/ABL1* (Abbot) were used.

**LTC-IC Assay.** The LTC-IC assay was performed essentially as previously described (20, 39) and is further outlined in *SI Materials and Methods*.

**Generation of KMT-1, a Polyclonal Rabbit Anti-Human IL1RAP Antibody.** Rabbits were immunized with the extracellular domain of IL1RAP obtained from R&D Systems. Serum from rabbits was purified according to standard procedures, except that an additional step was added in which antibodies binding to the Ig domain, present on the immunizing protein for increased half-life, were discarded through binding to Ig-loaded columns. Purified antibodies were confirmed by ELISA to bind the extracellular domain of IL1RAP and to be devoid of antibodies binding the human Ig domain. The polyclonal rabbit anti-human IL1RAP antibody was designated KMT-1 (Cantargia AB). When used in flow cytometry, a PE-conjugated goat anti-rabbit IgG antibody was used as the secondary reagent.

**ADCC Assay.** The ADCC assay was based on a protocol previously described (40). In brief, target cells were labeled with PKH26 (Sigma-Aldrich) according to manufacturer's instructions and either put directly into wells of a 96-well plate, or after sorting of $CD34^+CD38^-$ cells. The KU812 and KG-1 cell lines and primary $CD34^+$ cells were seeded at 10,000 cells per well, whereas primary $CD34^+CD38^-$ cells were seeded at 2,000 to 3,000 cells per well. Subsequently, antibodies were added to wells in different concentrations and incubated for 20 min before 100,000 NK effector cells were added to each well. NK cells were extracted from healthy volunteers after informed consent by using an NK-cell–negative cell isolation kit according to manufacturer's instructions (Miltenyi Biotec). Rabbit IgG antibodies purified from a nonimmunized rabbit were used as control in the experiments (R&D Systems). The next morning, the percentage of $7\text{-AAD}^+$ cells was measured using a FACS CANTO (Becton-Dickinson Immunocytometry Systems) to detect cell death. The average and SD of antibody-induced cell death was calculated according to the following equation: (Percentage $7\text{-AAD}^+$ cells$^{\text{at defined antibody concentration}}$ – Percentage $7\text{-AAD}^+$ cells$^{\text{no antibody}}$)/(0.01 × Percentage $7\text{-AAD}^-$ cells$^{\text{no antibody}}$) from at least three independent experiments.

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