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Antibodies targeting human IL1RAP (IL1R3) show therapeutic effects in xenograft models of acute myeloid leukemia

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Acute myeloid leukemia (AML) is associated with a poor survival rate, and there is an urgent need for novel and more efficient therapies, ideally targeting AML stem cells that are essential for maintaining the disease. The interleukin 1 receptor accessory protein (IL1RAP; IL1R3) is expressed on candidate leukemic stem cells in the majority of AML patients, but not on normal hematopoietic stem cells. We show here that monoclonal antibodies targeting IL1RAP have strong antileukemic effects in xenograft models of human AML. We demonstrate that effector-cell-mediated killing is essential for the observed therapeutic effects and that natural killer cells constitute a critical human effector cell type. Because IL-1 signaling is important for the growth of AML cells, we generated an IL1RAP-targeting antibody capable of blocking IL-1 signaling and show that this antibody suppresses the proliferation of primary human AML cells. Hence, IL1RAP can be efficiently targeted with an anti-IL1RAP antibody capable of both achieving antibody-dependent cellular cytotoxicity and blocking of IL-1 signaling as modes of action. Collectively, these results provide important evidence in support of IL1RAP as a target for antibody-based treatment of AML.

IL1RAP | AML | antibody | leukemia | immunotherapy

Acute myeloid leukemia (AML) is a hematologic malignancy with poor survival. Current treatment with chemotherapy does not target the leukemic cells specifically and is associated with severe side effects. Here we demonstrate that antibodies directed at the cell surface molecule IL-1 receptor accessory protein (IL1RAP), expressed on immature AML cells, show strong antileukemic effects in mice transplanted with human AML cells and that the mechanism behind the cell killing is through recruitment of effector cells. Using antibodies against IL1RAP also capable of blocking IL-1 signaling, we show that the proliferation of human AML cells can be inhibited, providing a second mode of action of IL1RAP antibodies. These results provide critical evidence in support of a rapid clinical development of an antibody-based anti-IL1RAP therapy in AML.

Significance

Acute myeloid leukemia (AML) is a hematologic malignancy with poor survival. Current treatment with chemotherapy does not target the leukemic cells specifically and is associated with severe side effects. Here we demonstrate that antibodies directed at the cell surface molecule IL-1 receptor accessory protein (IL1RAP), expressed on immature AML cells, show strong antileukemic effects in mice transplanted with human AML cells and that the mechanism behind the cell killing is through recruitment of effector cells. Using antibodies against IL1RAP also capable of blocking IL-1 signaling, we show that the proliferation of human AML cells can be inhibited, providing a second mode of action of IL1RAP antibodies. These results provide critical evidence in support of a rapid clinical development of an antibody-based anti-IL1RAP therapy in AML.
model of AML, we first used human immortalized MA9Ras cord blood cells coexpressing an MLL/AF9 fusion gene and an activated NRAS gene. MA9Ras cells express IL1RAP on the cell surface (Fig. 1A) and have been shown to faithfully recapitulate human AML in a xenograft setting (11). Untreated nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice engrafted with MA9Ras cells developed acute leukemia with a latency of 50–65 d and displayed high leukemic engraftment in bone marrow (BM) and spleen (Fig. S1A). In this model, approximately one-third of the mice normally develop extramedullary myeloid sarcoma consisting of leukemic tumors, that are infrequent in human AML patients (12). Treating mice with the anti-IL1RAP monoclonal antibody (mAb) 81.2 resulted in a significantly reduced frequency of leukemic cells in peripheral blood (PB) 35 d after transplantation, compared with the control group receiving an isotype control antibody (Fig. 1B). Platelet counts remained stable in mAb81.2-treated mice, suggesting improvement in hematopoietic parameters as a consequence of reduced leukemic burden (Fig. 1C). At day 62, all mAb81.2-treated mice showed low frequencies of leukemic cells in the PB, in contrast to controls (Fig. 1D). The mAb81.2-treated mice displayed a significantly prolonged survival (median 75 d, range 64–76) compared with control mice (median 64 d, range 62–67) and were killed due to MA9Ras-consisting myeloid sarcoma (Fig. 1E). Notably, despite being killed on average 11 d later than controls, the mAb81.2 treatment group had significantly reduced levels of leukemic cells in the BM (Fig. 1F). Moreover, mAb81.2-treated mice showed markedly reduced levels of leukemic cells in the spleen and lower spleen weights (Fig. 1G and Fig. S1B). Hematoxylin/eosin (H&E)-stained and human CD45-stained sections of BM and spleen revealed close to normal architecture in mAb81.2-treated mice, whereas the control mice displayed substantial infiltration of leukemic cells and disrupted architecture (Fig. 1H and Fig. S1C).

To validate IL1RAP as the specific target for the observed in vivo therapeutic effects, a second antibody, mAb3F8, was generated that binds to a different epitope on IL1RAP than mAb81.2 and with higher affinity. Similar to mAb81.2, treatment generated that binds to a different epitope on IL1RAP than mAb81.2, treatment showed almost no inhibitory effect in an IL-1 reporter assay, whereas mAb3F8 displayed potent inhibition of IL1R1 signaling in a dose-dependent manner (Fig. 2E). Neither of the antibodies affected IL1R1 signaling in the absence of IL-1, thereby excluding agonistic functions on IL-1–induced signaling (Fig. 2F). To investigate whether the MA9Ras cells were responsive to IL-1, suspension cultures were supplemented with IL-1, albeit no impact on cellular expansion or rate of apoptosis was observed (Fig. S3). Together, these results strongly suggest that the observed in vivo therapeutic effects in the MA9Ras model rely upon interactions with murine effector cells.

Anti-IL1RAP Treatment Shows Therapeutic Effect on Primary Human AML Cells in Vivo. To verify that the therapeutic in vivo effects from targeting IL1RAP in the MA9Ras model could be reproduced with primary AML cells, samples obtained from AML patients were transplanted into immunodeficient mice (Table S1).
followed by treatment with mAb81.2 or an isotype control antibody. Because effector cell function was found to be essential for the therapeutic effects in the MA9Ras model, we first transplanted three AML samples (AML1, AML2, and AML3) into NOD/SCID mice. Although it is challenging to obtain engraftment of primary AML cells in this strain, sample AML2 showed successful BM engraftment of leukemic cells, thereby allowing us to evaluate the therapeutic effect on primary human AML cells in vivo. Notably, the mAb81.2-treated mice displayed a significant reduction of BM leukemic cell frequency compared with controls (Fig. 3A and Fig. S4A). Increased engraftment of primary human AML cells can be obtained by using preconditioning and more severely immunocompromised mouse strains such as NSG (4). Hence, we next transplanted three samples (AML1, AML3, and AML4) into sublethally irradiated NSG or NSG SGM3 (NSGS) mice. Despite the less efficient effector-cell-mediated cell killing in these mice, mAb81.2 treatment of mice engrafted with sample AML4 resulted in significantly reduced levels of leukemic cells in BM and spleen (Fig. 3B). Treatment with mAb81.2 of samples AML1 and AML3 in vivo resulted in a similar trend (Fig. S4B and C). Collectively, these results demonstrate that antibodies targeting IL1RAP also show in vivo therapeutic effects on primary human AML cells.

**IL1RAP-Targeting Antibodies Mediate ADCC with Human NK Cells.** To investigate possible antibody-mediated effector-dependent mechanisms for cell killing in a human setting, chimeric mAb81.2 and mAb3F8 of human IgG1 subtype were analyzed for ADCC, antibody-dependent cellular phagocytosis (ADCP), and complement-dependent cytotoxicity (CDC) activity, by using human effector cells or complement. In agreement with previous results using primary human AML cells (9), MA9Ras cells were specifically killed by ADCC using human NK cells with both antibodies (Fig. 4A). By contrast, neither of the antibodies directed human macrophages to phagocytosis over the levels of an isotype control (Fig. 4B). Nor did the IL1RAP-targeting antibodies mediate specific cell death by CDC (Fig. 4C). The ability of the IL1RAP-targeting antibodies to mediate ADCC on different human AML cells was verified by using the human AML cell lines EOL1, MonoMac6, and OCI-AML1 (Fig. S5). Given that ADCC also could have an effect on normal tissues expressing IL1RAP, we next investigated the tissue expression of IL1RAP on an array of 30 different normal human tissues using mAb3F8. We have previously shown that, among normal blood cells, IL1RAP is expressed mainly on monocytes, and that primary AML cells are more sensitive to mAb81.2-mediated ADCC than corresponding normal BM cells (8, 9). We now show that IL1RAP staining was observed in only five tissues: BM, esophagus, lymph node, placenta, and spleen (Fig. S6 and Table S2). All together, these results suggest that antibody-based targeting of IL1RAP in humans would be associated with few side effects, although toxicity studies will be needed to confirm this finding.

**Anti-IL1RAP Antibody Suppresses Proliferation of IL-1–Dependent Human AML Cells.** Because mAb3F8 was found to block IL1R1 signaling in the IL-1 reporter assay in addition to mediating ADCC, we next explored whether this IL1RAP-targeting antibody could inhibit IL-1–induced proliferation of AML cells. Because MA9Ras cells are not responsive to IL-1, we used primary human AML cells. The response of human cells to murine IL-1 was found to be weak in these mice, mAb81.2 treatment of mice engrafted with sample AML4 resulted in significantly reduced levels of leukemic cells in BM and spleen (Fig. 3A). Collectively, these results demonstrate that antibodies targeting IL1RAP also show in vivo therapeutic effects on primary human AML cells.
AML cells (samples AML5, AML6, and AML7; Table S1) in serum-free medium with or without IL-1 followed by determination of the proliferation rate by incorporation of 5-ethynyl-2′-deoxyuridine (EdU). Samples AML5 and AML6 displayed increased proliferation after a 24-h stimulation with IL-1 (Fig. 4D). Treatment with mAb3F8 reduced the IL-1–induced cell proliferation by 25% (AML5) and 84% (AML6), compared with an isotype control antibody (Fig. 4E). In addition, in sample AML6, mAb3F8 inhibited the IL-1–induced cellular expansion by 55%, following a prolonged culture of 6 d (Fig. 4F). We also analyzed the expression of the proliferation marker Ki-67 on four IL-1–responsive primary human AML samples (AML5, AML6, AML8, and AML9; Table S1). Treatment with mAb3F8 inhibited the proliferative effect of IL-1 by 50%, as measured by expression of Ki-67 (Fig. 4G). These results demonstrate that an IL1R1-targeting antibody can reduce proliferation of primary human AML cells by interfering with IL1R1-signaling.

Discussion

Here, we provide in vivo evidence that antibodies targeting IL1RAP show therapeutic effects on human AML cells. The mechanism underlying the in vivo therapeutic effects was found to depend on interactions with effector cells. In IL-1–responsive primary AML samples, we identified antibody-induced suppression of IL-1 signaling as an additional mode of action.

Currently, only a few cell-surface markers have been shown to display preferential expression on AML stem cells compared with normal hematopoietic stem cells and with antibodies targeting these molecules demonstrating therapeutic efficacy in xenotransplantation models (24–27). However, apart from CD123, which showed limited efficacy in early clinical trials (28), to our knowledge, none has progressed to clinical evaluation in AML. IL1RAP is expressed on candidate AML stem cells, but not on corresponding normal cells (9). By using MA9Ras cells engrafted into immunodeficient mice, a strong antileukemic effect of the two independent IL1RAP-targeting antibodies mAb81.2 and mAb3F8 was observed in all hematopoietic compartments, including PB, BM, and spleen. Because the mice in the anti-IL1RAP treatment group were killed due to myeloid sarcoma formation, rarely seen in AML patients, the survival data poorly reflect the strong antileukemic effects observed in the BM, spleen, and PB. Importantly, the results were validated by using a xenograft model of primary human AML cells, where treatment with an anti-IL1RAP antibody significantly reduced the leukemic burden.

Consistent with the mechanisms of action for antibodies used to target cell-surface molecules in B-cell malignancies and in solid tumors (29–32), ADCC mediated by NK cells was found to be an important mode of action for killing of human AML cells. In contrast, neither of the anti-IL1RAP antibodies mediated specific cell death by ADCC or CDC of MA9Ras cells in vitro, suggesting that these mechanisms may be less critical for the observed effects. NK cells are considered the most important effector cell type in humans (30, 33, 34). The feasibility of an antibody-based immunotherapy depending on NK cells in AML is supported by reports showing that NK cell numbers are intact in AML patients (35) and that the levels of CD16, which activates NK cells for ADCC (36), is equal in AML patients and healthy subjects (37). Collectively, these data show that the effect of anti-IL1RAP immunotherapy in the MA9Ras xenograft model is mediated by effector cells and suggests that effector-cell–mediated mechanisms is an important and accessible mode of action in a future clinical setting.

Although MA9Ras cells share many properties with primary human AML cells, they do not respond to IL-1 stimulation and hence cannot be used for determining the impact of IL1RAP targeting antibodies on IL-1–dependent AML cell growth. In addition, because human cells respond poorly to murine IL-1, the therapeutic relevance of blocking IL-1 signaling cannot accurately be assessed in a murine xeno-transplantation setting.

![Figure 4](https://example.com/figure4.png)

**Fig. 4.** IL1RAP-targeting antibodies mediate ADCC with human NK cells, and mAb3F8 blocks proliferation of AML cells. (A) Frequency of dead MA9Ras cells with human NK cells in an ADCC assay (means ± SEM from technical duplicates; one representative experiment out of three). (B) Relative amount of human phagocytic macrophages in an ADCC assay with MA9Ras target cells. Data were normalized to 1.0 at conditions without antibody addition (means ± SEM from four experiments with technical duplicates in which the typical frequency of MA9Ras–macrophages were 20–50% without the addition of antibody). (C) Frequency of dead MA9Ras cells in a CDC assay using human normal serum (NS) or heat-inactivated serum (HIS) (means ± SEM from three experiments with technical duplicates). (D) Proliferation rate of three primary human AML samples with IL-1, as measured by incorporation of EdU. Data normalized to proliferation without IL-1 set to 1 (means ± SEM from one experiment with technical duplicates or triplicates). (E) Proliferation rate of samples AML5 and AML6 in the presence of IL-1 and mAb3F8 or isotype control antibody measured by EdU incorporation. Data were normalized to conditions without IL-1 set to 1 as indicated by the dotted line (means ± SEM from one experiment with technical duplicates or triplicates). (F) Total expansion of sample AML6 after 6 d of culture, with the dotted line indicating the number of cells without IL-1 (means ± SEM from one experiment with technical triplicates). (G) The effect of mAb3F8 or isotype control antibody on the IL-1–induced proliferation of primary human AML cells as measured by expression of the proliferation marker Ki-67. Data were normalized to conditions with IL-1 only set to 1 (means ± SEM from four AML patients with technical triplicates). P values were calculated with Student’s t test.
These features represent a limitation of our study because the effect of blocking IL-1 signaling with mAb3F8 could not be explored in vivo, and thus the relative impact of IL-1 signaling blockade on the total treatment effect could not be determined. To explore the effects of IL-1 signaling inhibition by an IL1RAP-targeting antibody, we instead performed in vitro investigations on primary cells harvested from AML patients. In agreement with previous reports using anti–IL-1 antibodies or the IL-1 receptor antagonist ILIRA, blocking IL-1 signaling with the IL1RAP-targeting antibody mAb3F8 significantly suppressed the proliferation of primary AML cells responsive to IL-1. The importance of IL-1 signaling in AML cells has recently obtained further support by a study that used a combined cytokine and siRNA screen against cell-surface receptors and identified IL-1 signaling inhibition as having the most dramatic effect on regulating growth of AML cells (38). Hence, based on available data, we propose that IL-1 signaling inhibition as a second mode of action in an antibody-based immunotherapy against IL1RAP should be important in the future treatment of AML.

Non-specific binding of a therapeutic antibody may lead to serious adverse effects. IL1RAP is expressed on candidate AML stem cells, but not on corresponding normal hematopoietic stem cells (9, 10). Among normal blood cells, IL1RAP is expressed mainly on monocytes, and AML cells are more sensitive than corresponding normal BM cells to ADCC induced by IL1RAP-targeting antibodies (8, 9). Here, absent or weak IL1RAP expression was shown in a majority of normal human tissues. Collectively, these data suggest a low risk of adverse effects of an antibody-mediated immunotherapy against IL1RAP in AML; however, toxicology studies using animal species with cross-reactivity for the IL1RAP-targeting antibodies will be needed to provide a better prediction.

In summary, we demonstrate that IL1RAP provides a therapeutic target in AML and that the dual mode of action of IL1RAP-targeting antibodies, combining effector-cell–mediated mechanisms with IL-1–signaling suppression, is predicted to be beneficial. These findings strongly support a rapid clinical development of an antibody-based IL1RAP therapy in AML.

Materials and Methods

Culturing and Isolation of Cells. MA9Ras cells were previously generated by retroviral transduction of primitive cord blood cells with a ML1A9 fusion gene and an activated NRAS gene (11). Cells were grown in Iscove’s modified Dulbecco’s medium supplemented with 10% (vol/vol) FBS and 1% penicillin/ streptomycin/glutamine and with a 1:1,000 volume of mercapto-ethanol added freshly. Cell lines EOL1, MonoMac6, and OGI-AML1 were cultured according to the supplier’s instructions (DSMZ). For primary cells, patients diagnosed with AML donated BM or PB after informed consent. The protocol was approved by the Institutional Ethics Committee and was in accordance with the Declaration of Helsinki. Mononuclear cells (MNCs) were isolated by density gradient separation.

IL1RAP-Targeting Antibodies. The antibodies mAb81.2 and mAb3F8 were generated by standard hybridoma technique as described (9).

In Vivo Antibody Treatment. All animal experiments were approved by The Swedish Board of Agriculture, Malmö/Lund animal ethics committee in Lund, Sweden. Unconditioned NOD/SCID (NOD.CB17- SCID/Slc ) mice serially engrafted with human AML MNCs were transplanted into unconditioned NOD/SCID mice or sublethally irradiated (200 rad) NSG or NSG mice. Treatment was initiated 3 d after transplantation and administered by biweekly i.p. injections with mAb81.2 or mlgG2a isotype control antibody of 100 μg per dose, corresponding to 4 mg/kg, for all doses except for the first dose, which was 500 μg. Experiments were terminated at 28 d after transplantation for NOD/SCID mice and 35–57 d after transplantation for NSG/NSG mice.

Sample Preparation and Flow Cytometry. BM cells from mice were isolated by flushing of one femur or by crushing femurs, tibiae, and the pelvis. Spleens were mashed through a 70-μm mesh filter. PB was prepared for flow cytometry by ammonium chloride treatment to remove red cells. The following antibodies (all anti-human) were used in flow cytometry analysis: CD45-APC (BD Biosciences), CD33-BV421, CD33-PECy7, CD19-PerCP-Cy5.5, CD3-PECy7 (BioLegend), and IL1RAP-PE (R&D Systems). In the in vivo experiments with primary human AML cells, CD45-CD33+ cells were considered to be leukemic. For all flow-cytometric analysis, a FACs Canto (BD) was used.

Total Cell Expansion. MA9Ras cells were seeded in technical duplicates at a density of 10,000 cells in a volume of 100 μL in normal culture medium with or without the addition of 10 μg/mL mAb81.2, mAb3F8, or a mlgG1 isotype control antibody for 48 h. For primary AML cells, 20,000 cells were plated in 100 μL of serum-free medium in technical triplicates and cultured for 6 d. The total cell numbers were determined with CountBright Absolute Counting Beads (Molecular Probes) and flow cytometry.

Apoptosis. For analysis of apoptotic cells, 50,000–75,000 MA9Ras cells were seeded in technical duplicates and cultured for 48 h, followed by detection of viable, apoptotic, or dead cells by staining with Annexin V and 7-aminoactinomycin D (7AAD). Samples were analyzed by flow cytometry.

Proliferation. Primary human AML MNCs were seeded in technical duplicates or triplicates at a density of 100,000 cells per well in a volume of 100 μL of serum-free medium with or without the addition of 0.5–10 ng/mL IL-1α and 10 ng/mL mAb3F8 or a corresponding mlgG1 isotype control. Proliferation was determined by measuring the incorporation of EdU after 24 h with Click-iT EdU Flow Cytometry Assay Kit (Molecular Probes) according to manufacturer’s instructions. Expression of the proliferation marker Ki-67 was determined after 48 h of culture, by using a Ki-67–APC antibody (Miltenyi Biotec) upon fixation and permeabilization of the cells with 1.6% paraformaldehyde and ice-cold 95% ethanol.

ADCC Assay. Target cells were labeled with PKH26 (Sigma-Aldrich) according to the manufacturer’s instructions and seeded in technical triplicates. After 1 h at 37 °C, cells were incubated with 0.5–10 μg/mL mAb81.2, mAb3F8, or a corresponding hlgG1 isotype control, human NK cells were added at a 10:1 effector-to-target cell (E/T) ratio, and plates were incubated overnight. The following day, the percentage of dead target cells (PKH26+/EdU−) was measured by flow cytometry.

ADCP Assay. Human monocytes were isolated from PB and matured into macrophages by addition of M-CSF to the culture medium. Proper maturation was confirmed by morphology and flow cytometry analysis after staining for CD206. Mature macrophages were seeded at a density of 40,000 cells per well and allowed to adhere overnight. Target MA9Ras cells were stained with PKH26, preincubated with 10 μg/mL mAb81.2, mAb3F8, or a corresponding hlgG1 isotype control antibody for 30 min, and added at a 1:1 E/T ratio to the wells in technical duplicates. After incubation for 2 h at 37 °C, cells were detached, and the amount of PKH26-positive macrophages was determined by using flow cytometry. At this E/T ratio, there was a variation of 20–50% MA9Ras–macrophages without antibody addition between the individual experiments. Thus, at presentation, data were normalized to 1.0 relative amount of MA9Ras–macrophages without antibody addition.

CDC Assay. MA9Ras cells were seeded in technical duplicates at a density of 100,000 cells per well in serum-free StemSpan medium (Stem Cell Technologies). Cells were preincubated with 10 μg/mL mAb81.2, mAb3F8, or a corresponding hlgG1 isotype control for 30 min. Native or heat-inactivated human serum was added to a final concentration of 10% or 50%. After incubation for 2 h at 37 °C, cells were stained with ToPro3-iode and analyzed by flow cytometry.
IL-1 Signaling Reporter Cell Assay. HEK-Blue IL-1β cells (Invivogen) were harvested and plated in technical duplicates at a density of 50,000 cells per well in a 96-well plate. MAbr1.2, mAb38F, or a corresponding mAbG1 control antibody was added to the wells in a concentration range of 0.01–10 μg/mL. After preincubation for 30 min, IL-1β was added to a final concentration of 0.5 ng/mL, and the plate was incubated overnight. To ensure assay functionality, one set of samples was pretreated with 10 ng/mL IL1RA (R&D Systems). To examine whether any of the antibodies were able to induce IL-1R activation in the absence of IL-1β, samples were incubated with 10 μg/mL antibody without addition of the ligand. The following day, substrate was added to the supernatants, and samples were analyzed for absorbance at 620 nm. For measuring the response to murine IL-1, the cells were stimulated with 0.1 pg to 10 ng of human or murine IL-1 in the absence of antibody.

Immunostaining of Normal Tissue Array. Immunohistochemistry on normal tissue arrays was performed at MicroMorph AB (Lund). Briefly, a tissue array containing 30 different normal human tissues from three individuals was immunostained. Overexpression of an HRP-conjugate control antibody. High IL1RAP-expressing SKMEL-S cells were used as positive control and KG1 cells as negative. For detection, an HRP-conjugated anti-mouse antibody (Dako)

was used. The staining intensity was determined as follows: negative (0), weak staining (1+), moderate staining (2+), or strong staining (3+).

Statistical Analyses. GraphPad Prism software (GraphPad Software) was used for statistical analyses. Statistical differences were determined to be significant at P < 0.05 using two-tailed Student’s t test (MAbRas xenograft data and all in vitro data), two-tailed Mann–Whitney log-rank test (xenograft data using primary AML cells), or Mantel Cox log rank test (survival). In vivo data are presented as mean ± SD or median ± interquartile range, with the value for each individual mouse represented as a symbol. In vitro data are presented as mean ± SEM.

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