

Disruption of CSF-1R signaling inhibits growth of AML with inv(16)

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Key Points

- Human inv(16) AML cells express CSF-1R and are exposed to CSF-1 *in vivo*.
- Inhibition of CSF-1R signaling reduces viability of inv(16) AML cells *in vitro* and in therapeutic settings in humanized mice *in vivo*.

Introduction

Inversion of chromosome 16 [inv(16)(p13q22)], resulting in the fusion transcript *CBFB-MYH11*, is a chromosomal rearrangement recurrently found in acute myeloid leukemia (AML).^{1,2} Although AML with inv(16) has a comparatively favorable outcome upon standard chemotherapy, almost half of patients eventually relapse. Thus, a better understanding of disease pathophysiology and improved treatments are necessary.³ We recently demonstrated that human inv(16) AML cells express colony-stimulating factor 1 receptor (CSF-1R) and that human colony-stimulating factor 1 (CSF-1) was required for high-level *in vivo* engraftment of inv(16) AML cells in immunodeficient mice.^{4,5} Here, we consequently tested whether CSF-1 can be measured in inv(16) AML patient sera and if inhibition of CSF-1R signaling can selectively inhibit inv(16) AML growth *in vitro* and *in vivo*.

Methods

AML cells were obtained upon written informed consent from patients with newly diagnosed AML (Cantonal Ethics Committee of Zurich; KEK-ZH-Nr. 2009-0062/1). AML karyotypes and mutational status are provided in supplemental Table 1. Mononuclear cells (MNCs) were purified by density gradient centrifugation. CD3- and CD19-expressing cells were depleted by immunomagnetic separation. AML cells were cultured in Iscove modified Dulbecco medium supplemented with 20% fetal bovine serum, 1% bovine serum albumin, 2 mM L-glutamine, 50 μ M 2-mercaptoethanol, antibiotics, human interleukin-3 (IL-3; 20 ng/mL), IL-6 (10 ng/mL), IL-11 (10 ng/mL), granulocyte-macrophage colony-stimulating factor (50 ng/mL), stem cell factor (10 ng/mL), Flt3 ligand (10 ng/mL), and thrombopoietin (50 ng/mL) \pm CSF-1 (20 ng/mL). Humanized cytokine knockin mice (MISTRG) were generated as reported previously.⁶ Newborn mice were sublethally irradiated with 1×150 cGy. A total of 1×10^6 primary AML cells (in 20 μ L phosphate-buffered saline) were injected intrahepatically using a 30-G needle.^{4,7} Mice were treated and analyzed as indicated in supplemental Methods.

Results and discussion

To determine if CSF-1 is measurable in inv(16) AML patients and thus CSF-1R on inv(16) AML cells might be stimulated via this pathway, we analyzed CSF-1 levels in plasma from inv(16) AML patients ($n = 13$), non-inv(16) AML patients ($n = 37$), and patients without a hematological disease ($n = 15$). CSF-1 levels varied widely, with a mean concentration of 400 pg/mL in the inv(16) AML cohort, 139 pg/mL in the non-inv(16) AML cohort, and 130 pg/mL in patients without a hematological disease (Figure 1A), although these differences were not statistically significant. In line with prior results, we detected a relative upregulation of CSF-1R in bone marrow cells of inv(16) AML, but not in other genetically defined AML types, using microarray data from an international cohort study ($n = 536$)

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For original data, please contact the corresponding author (markus.manz@usz.ch). Gene expression data are publicly available (ArrayExpress; accession number: E-MTAB-3444).

The full-text version of this article contains a data supplement.

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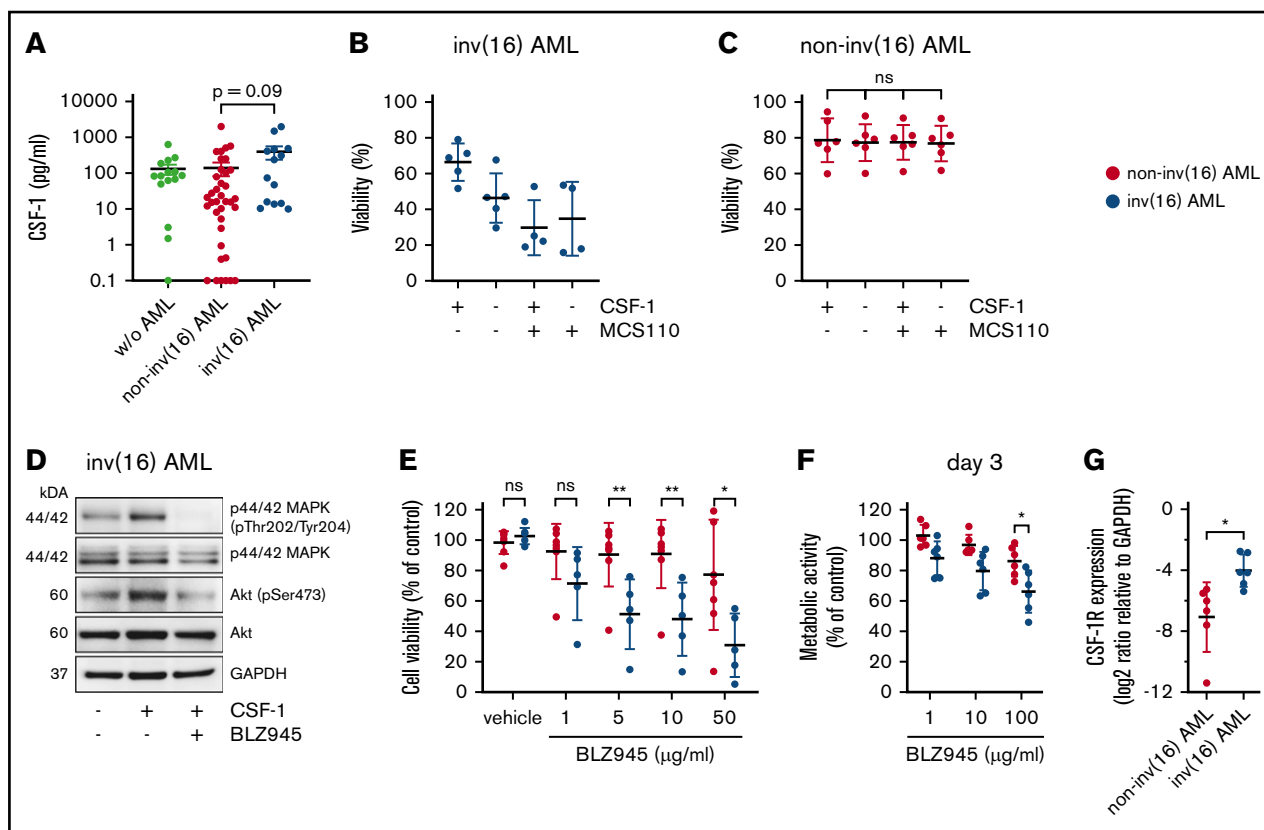


Figure 1. CSF-1R signaling promotes and signaling-inhibition decreases survival of inv(16) AML cells in vitro. (A) Plasma from peripheral blood was collected from inv(16) AML patients ($n = 14$), non-inv(16) AML patients ($n = 37$), and patients without a hematological disease ($n = 15$). AML samples were collected at first diagnosis or relapse, and CSF-1 concentration in blood was measured by enzyme-linked immunosorbent assay. Graph shows mean \pm standard error of the mean. (B) Analysis of viability of different primary inv(16) AML ($n = 5$) (B) and non-inv(16) AML ($n = 6$) (C) samples (CD3/CD19 depleted) after incubation for 10 days with or without CSF-1 and MCS110 (40 $\mu\text{g}/\text{mL}$), a monoclonal blocking anti-CSF-1 antibody, respectively. Viability was determined by flow cytometry. (D) Western blot analysis of CSF-1R downstream signaling pathways, including phospho-p44/42 MAPK (Thr202/Tyr204) and phospho-Akt (Ser473), in primary AML with inv(16) cells in response to CSF-1 and the CSF-1R inhibitor BLZ945. Before incubation with CSF-1 (30 minutes) and BLZ945 (200 $\mu\text{g}/\text{mL}$) (2 hours), cells were starved in cytokine-free medium for 2 hours. GAPDH and total p44/42 MAPK/Akt are included as loading controls (experiment representative of $n = 3$). (E) Primary inv(16) AML ($n = 5$) and non-inv(16) AML ($n = 7$) samples (CD3/CD19 depleted) were cultured for 10 days in the presence of CSF-1 (20 ng/mL) with different concentrations of BLZ945 or a vehicle control (Captisol 20%) or left untreated. Viability was analyzed by flow cytometry. Data indicate viability of primary AML cells relative to untreated cells (control). (F) Metabolic activity of primary inv(16) AML ($n = 6$) and non-inv(16) AML ($n = 6$) in response to different concentrations of BLZ945 determined by MTT assay at day 3. Changes are calculated relative to untreated cells. (G) CSF-1R expression analysis by quantitative polymerase chain reaction of non-inv(16) ($n = 6$) and inv(16) ($n = 6$) AML samples. Graph indicates the \log_2 of the ratio of the Ct values of *CSF1R* to the reference gene *GAPDH* for each sample. Graphs show mean \pm standard error of the mean. * $P < .05$; ** $P < .01$; *** $P < .001$. ns, not significant.

(supplemental Figure 1A; supplemental Table 2).⁸ Interestingly, while strong CSF-1R upregulation was unique to inv(16) AML, the data also demonstrate overexpression of other cytokine receptors in different genetically defined AML subgroups, indicating their possible biological roles in those AML entities.⁹

To test whether CSF-1 is of relevance for inv(16) AML cell survival in vitro, we cultured primary human inv(16) AML cells with a mixture of hematopoietic progenitor cell supporting cytokines at saturating doses, with or without the addition of CSF-1. While AML cells showed a certain degree of maturation, immature surface markers were maintained during the 10-day culture period (supplemental Figure 1B). Importantly, inv(16) AML cells incubated without CSF-1 showed a significantly lower viability when compared with those

incubated with CSF-1, and positive effects of CSF-1 were abrogated by MCS110, a monoclonal CSF-1 neutralizing antibody (Figure 1B). In stark contrast, we observed no impact on in vitro survival of non-inv(16) AML samples by the addition of CSF-1 and its neutralization via MCS110 (Figure 1C).

We then evaluated inhibition of CSF-1R signaling via the selective tyrosine kinase inhibitor BLZ945. As expected, inhibition of CSF-1R resulted in reduced phosphorylation of downstream targets p44/42 MAPK (ERK-1/2) and Akt (Figure 1D).¹⁰ In line with these findings and supporting the importance of this signaling pathway in inv(16) AML, transcriptomic analysis showed an enhanced positive regulation of the MAPK/ERK pathway in inv(16) AML compared with other AML subtypes (supplemental Figure 1C). Incubation of primary inv(16) AML cells with BLZ945 resulted in a reduced

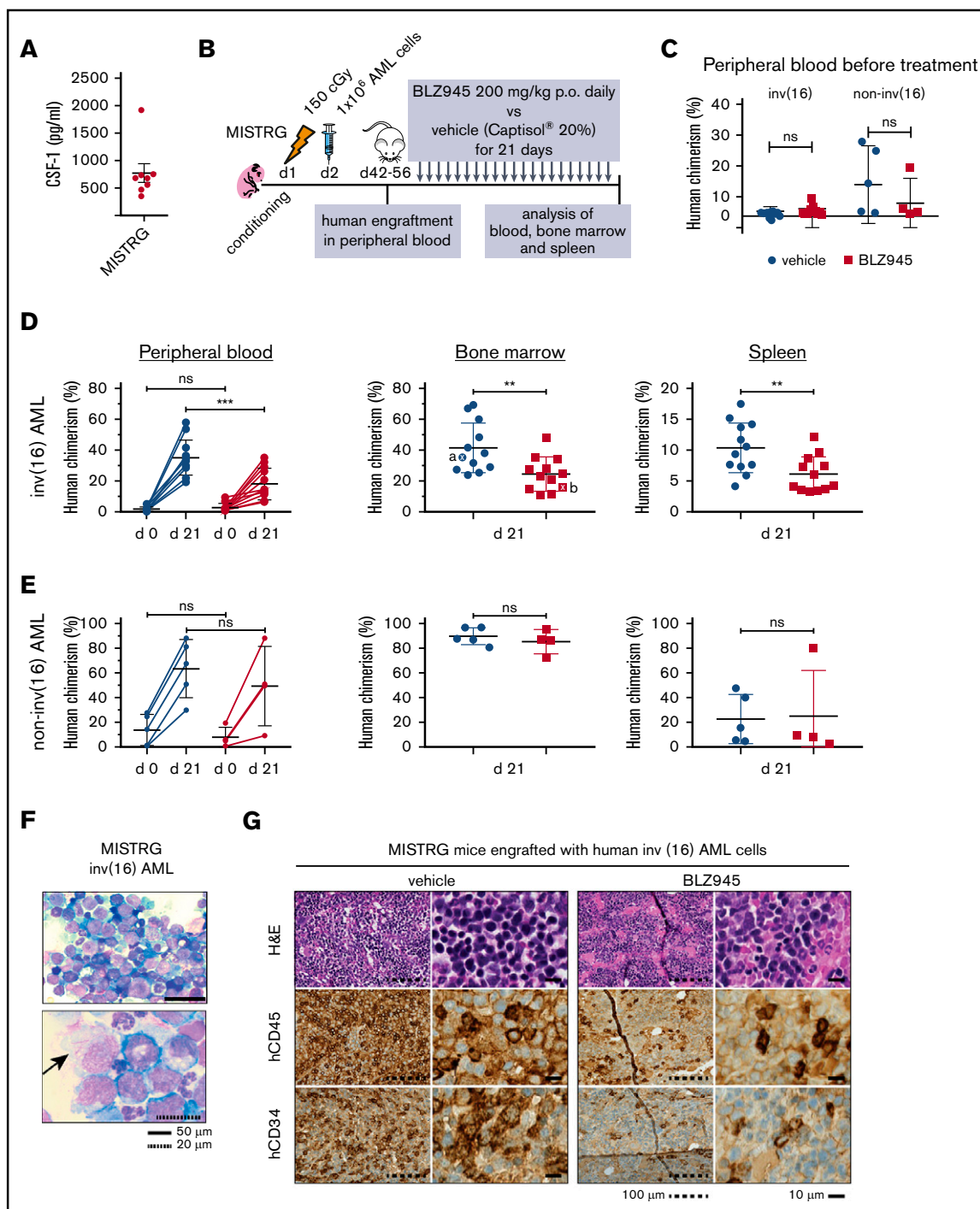


Figure 2. CSF-1R inhibition reduces inv(16) AML growth in a therapeutic xenograft model. (A) Plasma from adult MISTRG ($n = 8$) was collected, and CSF-1 concentration was measured by enzyme-linked immunosorbent assay. (B) Experimental scheme: 1×10^6 primary AML cells (inv(16), $n = 4$ different patients; non-inv(16), $n = 3$) were transplanted intrahepatically into irradiated newborn mice (MISTRG). Upon detection of human blasts in peripheral blood, the CSF-1R inhibitor BLZ945 (200 mg/kg) or a vehicle (equivalent amount Captisol 20%) was orally administered daily for 21 days. AML engraftment was determined in blood prior to treatment and in blood, bone marrow, and spleen after treatment. (C) AML engraftment (human CD45⁺ cells of total CD45⁺ cells) of the vehicle control (blue) and BLZ945 (red) cohort prior to treatment. (D-E) Human inv(16) AML (D) and non-inv(16) AML (E) engraftment (human CD45⁺ cells of total CD45⁺ cells) of the vehicle control (blue) and BLZ945 (red) cohort in peripheral blood (left) (day 0 [d0], start of treatment), bone marrow (middle), and spleen (right) after 21 days (d21) of application (inv(16) AML, $n = 24$; non-inv(16), $n = 9$). (F) Demonstration of myeloblasts and multiple intracellular Auer rods (black arrow) in a myeloid leukemic blast in the bone marrow of a MISTRG mouse engrafted with human AML with inv(16) cells (Giemsa stain). (G) Representative images of bone marrow histology of inv(16) AML engrafted MISTRG mice, detecting human CD45 and CD34. Small letters in panel C (vehicle = a; BLZ945 = b) point out engraftment levels measured by flow cytometry in the bone marrow of the animals shown. Graphs show mean (black bar) \pm standard error of the mean. * $P < .05$; ** $P < .01$; *** $P < .001$. H&E, hematoxylin and eosin.

viability and increased apoptotic cell death when compared with non-inv(16) AML cells (Figure 1E; supplemental Figure 2A-C). Correspondingly, incubation of inv(16) AML cells with BLZ945 led to a reduced metabolic activity as detected by MTT assay at day 3 and day 6 compared with non-inv(16) cells (Figure 1F; supplemental Figure 2D). In contrast, *in vitro* treatment of non-inv(16) AML with BLZ945 showed no statistically significant effect on cell viability compared with untreated cells at low BLZ945 concentrations, but only at doses ≥ 100 $\mu\text{g/mL}$ (supplemental Figure 2E-G). BLZ945 at higher doses might inhibit other relevant tyrosine kinases, notwithstanding its relatively high target specificity, a hypothesis supported by the finding that non-inv(16) AML was neither stimulated by CSF-1 nor inhibited by CSF-1 neutralization (Figure 1C). Also, higher concentrations of BLZ945 showed cytotoxic effects on healthy human bone marrow cells, potentially indicating on- and off-target reactivity of this substance (supplemental Figure 2H). To further reveal mechanisms of CSF-1 dependence and possibly predict the response to CSF-1R inhibition, we analyzed CSF-1R expression levels in primary AML cells by quantitative polymerase chain reaction. As expected, inv(16) AML cells showed increased expression levels compared with non-inv(16) (Figure 1G). A subanalysis revealed a correlation of CSF-1R expression levels and *in vitro* response rates to BLZ945 (supplemental Figure 2I). Moreover, we analyzed CSF-1 serum levels of patients at diagnosis and response rates to BLZ945. Here, we could observe the tendency that primary AML cells from patients with high CSF-1 serum levels showed increased cytotoxicity in response to BLZ945 treatment *in vitro* (supplemental Figure 2J).

To test the effectiveness of BLZ945 on inv(16) AML growth inhibition *in vivo*, we established a therapeutic xenograft model using Rag2-, Il2rg-deficient mice, in which mouse cytokines (macrophage colony-stimulating factor/CSF-1, IL-3, granulocyte-macrophage colony-stimulating factor, and thrombopoietin) were replaced by their human counterparts at the respective mouse loci.⁶ Of note, these mice have similar human CSF-1 plasma levels as human inv(16) AML patients (average concentration of 767 pg/mL) (Figures 1A and 2A). Mice were transplanted with patient-derived inv(16) AML or non-inv(16) AML cells. Upon detection of AML blasts in peripheral blood, mice were allocated to 2 groups, matched by leukemia engraftment levels. Animals were subsequently treated daily with BLZ945 (200 mg/kg) or control Captisol 20% (vehicle) orally for 21 days (Figure 2B-C). BLZ945 was well tolerated, with no weight loss and without impaired mouse hematopoiesis in both MISTRG and wild-type mice (supplemental Figures 3A-G and 4A-E). Human AML engraftment in mice was analyzed upon termination by flow cytometry and defined as percentage of human CD45⁺ of all CD45⁺ cells (human and mice) (Figure 2C, supplemental Figure 4F). BLZ945 treatment resulted in significantly reduced growth of inv(16) AML in bone marrow, blood, and spleen compared with the vehicle control group (Figure 2D; supplemental Table 3). By testing a small cohort of mice transplanted with non-inv(16) AML primary cells, our data indicate a lower sensitivity of non-inv(16) AML cells to BLZ945 *in vivo* (Figure 2E). The data obtained by flow cytometry were also verified by cytology (Figure 2F) and bone marrow histology analysis (Figure 2G). However, further studies are required to clarify the susceptibility of different AML subtypes to CSF-1R inhibitors as a single agent or in combination therapies.

In summary, we demonstrate significantly reduced viability of inv(16) AML cells *in vitro* and *in vivo* in response to CSF-1R signaling inhibition. Importantly, xenograft studies in humanized MISTRG mice closely reflect exposition of AML to CSF-1 in patients, thus suggesting that the inhibitory effect of BLZ945 could indeed also apply in a clinical setting. However, while inv(16) AML growth was significantly reduced by CSF-1R signaling inhibition, a relevant portion of inv(16) AML cells survived treatment during the experimental observation time. Thus, not surprisingly, CSF-1R inhibition is unlikely to be sufficiently effective as single-agent therapy. It is important to point out in this context that midostaurin, the first US Food and Drug Administration–approved inhibitor of the recurrently mutated and constitutively activated membrane tyrosine kinase FLT3, also primarily showed a growth deceleration rather than an eradication of AML cells in a xenograft model.¹¹

Concurrently with our data, a recent study showed that CSF-1R inhibition reduces *in vitro* cell viability in >20% of AML patient samples due to inactivation of paracrine signals by supportive cells.¹² Interestingly, the CSF-1R inhibitor (GW2580) used in this study was found to inhibit preferentially *de novo* and favorable-risk (European LeukemiaNet classification) AML patient samples.

Independently of AML, the use of CSF-1R inhibitors demonstrated a favorable safety profile in patients with solid tumors.¹³ Currently, 2 clinical trials are recruiting to test the safety, tolerability, and efficacy of BLZ945 in nonhematological diseases (NCT02829723 and NCT04066244). The first results revealed a tolerable safety pattern without severe hematotoxic side effects.¹⁴

However, CSF-1R inhibition in cancer has to be evaluated carefully, as unexpected effects may occur. In a murine model of spontaneous metastasis from primary Lewis lung carcinoma tumors in immunocompetent mice, inhibition of CSF-1R increased the risk of developing metastatic disease by reducing the number of natural killer cells due to a lack of myeloid cells that provide IL-15.¹⁵ Interestingly, administration of exogenous IL-15 during treatment restores natural killer cell numbers and metastasis control.

In summary, CSF-1R inhibition might be a promising target in AML treatment of specific subgroups like AML with inv(16) and should be evaluated in further studies.

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Authorship

Contribution: A.S. and N.F.R. contributed samples, performed experiments, analyzed data, and wrote the manuscript; J.M. performed experiments and analyzed data; C.M.W. contributed samples,

performed experiments, and analyzed data; M.H.E.W. analyzed data; R.M. performed experiments; N.W.-V.v.W. performed experiments; P.J.M.V. analyzed data; R.M., S.B., and A.P.A.T. contributed samples and discussed data; and M.G.M. directed the study and wrote the manuscript.

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