Targeting Interleukin-2 to the Bone Marrow Stroma for Therapy of Acute Myeloid Leukemia Relapsing After Allogeneic Hematopoietic Stem Cell Transplantation

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Abstract

The antibody-based delivery of interleukin-2 (IL2) to extracellular targets expressed in the easily accessible tumor-associated vasculature has shown potent anti-leukemic activity in xenograft and immunocompetent murine models of acute myeloid leukemia (AML), especially in combination with cytarabine. Here, we report our experiences in four patients with relapsed AML after allogeneic hematopoietic stem cell transplantation (allo-HSCT), who were treated with the immunocytokine F16-IL2, in combination with low-dose cytarabine (LDAC). One patient with disseminated extramedullary AML lesions achieved a complete metabolic response in PET/CT, which lasted three months. Two out of three patients with bone marrow (BM) relapse achieved a blast reduction with transient molecular negativity. One of the two patients enjoyed a short complete remission before AML relapse occurred two months after the first infusion of F16-IL2. In line with a site-directed delivery of the cytokine, F16-IL2 led to an extensive infiltration of immune effector cells in the BM. Grade 2 fevers were the only non-hematologic side effects in two patients. Grade 3 cytokine-release syndrome developed in the other two patients, but was manageable in both cases with glucocorticoids. The concept of specifically targeting IL2 to the leukemia-associated stroma deserves further evaluation in clinical trials, especially in patients who relapse after allo-HSCT.
Introduction

Therapeutic approaches interfering with tumor angiogenesis have become fully incorporated into clinical practice nowadays (1). In addition to anti-angiogenesis, a second, conceptually different strategy, “vascular targeting”, takes advantage of tumor vessels for therapeutic purposes and aims at the selective pharmacodelivery of therapeutic payloads (e.g., drugs, cytokines, radionuclides) to the tumor site by their conjugation to carrier antibodies, which specifically home to tumor-associated vasculature (2-5). The approach benefits from the intrinsic accessibility of vascular targets and bypasses many of the hurdles associated with the targeting of cancer cell markers, such as physical and kinetic barriers of bulky tumors or antigen heterogeneity among tumor cells. Furthermore, whereas cancer cell surface proteins are in many cases shared by normal cells, vascular and stromal targets are available, which allow a clear-cut discrimination between healthy and neoplastic tissues (2,3). While it was initially assumed that vascular-targeting antibodies would mainly be relevant for solid tumors, we recently discovered that well-characterized vascular targets are abundantly expressed in hematologic malignancies such as Hodgkin and non-Hodgkin lymphomas, allowing the antibody-mediated deposition of bioactive payloads at the lymphoma site (6,7).

In 2000, we reported that acute myeloid leukemia (AML) is associated with extensive neovessel formation in the bone marrow (BM) (8). In an attempt to evaluate a vascular-targeting strategy in AML, we have recently shown that the alternatively spliced extra-domains A (EDA-Fn) and B (EDB-Fn) of fibronectin and the extra-domain A1 of tenascin-C (TnC-A1) are abundantly expressed in AML BM and extramedullary AML lesions (5). These markers are almost exclusively found in the tumor-associated vasculature and stroma while being virtually absent in normal organs and have been shown to be suited for site-specific pharmacodelivery approaches (2,3,9,10). In immunocompromised and immunocompetent mouse models of myelosarcoma-type AML, the antibody-mediated delivery of the pro-inflammatory cytokine interleukin-2 (IL2) to the leukemia-associated vasculature led to substantial leukemia growth retardation in a process mediated by CD8$^+$ T cells and natural killer (NK) cells, while equivalent doses of non-targeted IL2 were ineffective (5). Most importantly, the combination of the immunocytokine with cytarabine promoted complete leukemia eradications.

Here, we report the use of the immunocytokine F16-IL2, which mediates the selective delivery of human IL2 to TnC-A1, in combination with low-dose cytarabine
(LDAC) in heavily pre-treated AML patients with relapsed or refractory disease after allogeneic hematopoietic stem cell transplantation (allo-HSCT).
Case reports

**Patient 1** was a female patient who was diagnosed with complex karyotype AML FAB M1 in February 2007 at 45 years of age. Her detailed medical history has been described previously (5). Briefly, in May 2013, the patient ultimately presented with rapidly progressing, disseminated myelosarcoma (chloroma) nodules after multiple lines of intensive therapy, including two unrelated donor allo-HSCTs, while the BM was free of leukemia. [18F]2-fluoro-2-deoxy-D-glucose positron emission tomography/computed tomography (PET/CT) revealed hypermetabolic manifestations in the left deep cervical area, in the mediastinum, in the hilum of the liver (leading to cholestasis), in the mesentery, in the hilum of the right kidney, in the sternum, in the transverse process of the 9th thoracic vertebra, and subcutaneously in the left gluteal area.

50-year-old female **Patient 2** had a diagnosis of NPM1-mutated, normal karyotype AML FAB M1 in August 2010. Induction therapy with two cycles of HAM resulted in a first CR and was followed by consolidation with TAD and maintenance therapy according to the AMLCG 2008 protocol. In June 2011, the first AML relapse occurred and she underwent matched related donor allo-HSCT, leading to a second CR. Ten months later, the second AML relapse occurred and another allo-HSCT from a matched unrelated donor was performed after chemotherapy with amsacrine, fludarabine and high-dose cytarabine (FLAMSA) and conditioning with melphalan, busulfan, fludarabine and ATG. The second allo-HSCT resulted in another CR, but a third AML relapse was diagnosed nine months later.

**Patient 3** was a male 53-year-old patient who was diagnosed with AML secondary to chronic myelomonocytic leukemia (CMML). CMML was first diagnosed in August 2011 and was treated with azacytidine until August 2012, when the disease transformed to AML. While he was refractory to HAM induction therapy, he received allo-HSCT from a matched unrelated donor in December 2012 after conditioning with melphalan, fludarabine, ATG and 8 Gy TBI and achieved a CR which lasted six months. However, AML relapse was diagnosed in July 2013.

In January 2007, NPM1-mutated AML FAB M5 with normal karyotype was diagnosed in **Patient 4** at the age of 39 years. After induction with two courses of DA he achieved a CR, followed by consolidation with three cycles of high-dose cytarabine. In May 2011, the first AML relapse occurred (now FLT3-ITD positive), and the patient was admitted to allo-HSCT with a matched unrelated donor after chemotherapy with HAM and conditioning with cyclophosphamide, ATG and 12 Gy TBI in July 2011, leading to a
second CR of two years. In September 2013, NPM1-positive, FLT3-ITD-negative AML relapse was diagnosed with the karyotype 46,XY,t(2;3)(q?33;p15)[7]/46,XY[13]. At that time, the patient was reluctant to receive intensive chemotherapy followed by a second allo-HSCT.
Methods

Patients and treatment

From June 2013 to December 2013, four AML patients who relapsed after multiple chemotherapies and one (n = 2) or two (n = 2) allo-HSCT were treated in a compassionate use setting with F16-IL2 and LDAC at the University Hospital Muenster, Germany. The joint ethical board of the University of Muenster and the regional Physician's Chamber of Westfalen-Lippe was consulted for each individual case. Written informed consent from each patient was obtained in accordance with the Declaration of Helsinki. F16-IL2, consisting of the human anti-TnC-A1 antibody F16 fused to human IL2, was provided by Philogen (Siena, Italy). F16-IL2 was administered weekly as a 3-hour intravenous infusion. Patients received 30 Mio IU IL2 equivalents on the first infusion and 50 Mio IU in the following infusions when the first was well tolerated. LDAC was administered subcutaneously on days 1-10 at 5 mg twice daily. Except patient 1, who required low-dose systemic steroids for chronic skin GVHD, none of the other three patients received immunosuppressive medication when post-allo-HSCT AML relapse was diagnosed and treatment with F16-IL2 and LDAC was initiated. A Phase Ib study with F16-IL2 in combination with chemotherapy in patients with solid tumors is ongoing and has shown that doses up to 70 million IU can be safely administered (NCT01134250, NCT01131364).

Histochemical analyses

For analysis of TnC-A1 expression, cryo-sections of pre-therapeutic BM biopsies were stained with biotinylated F16 as described (5). Antibody detection was performed with streptavidin Alexa Fluor 488 (Invitrogen, Darmstadt, Germany). For analysis of in vivo targeting, F16-IL2 bound to its target in situ was detected in post-therapeutic BM specimens using the LSAB (labeled streptavidin-biotin) method. Sections of formalin-fixed paraffin-embedded BM biopsies were prepared, followed by antigen retrieval in citrate buffer (pH 6, 30 minutes, 99°C). Sections were incubated with a rabbit anti-human IL2 antibody (1:125, Abcam, Cambridge, UK) overnight at 4 °C. Visualization was performed using Dako REAL Detection System/AP following the manufacturer’s instructions. Samples were analyzed on a Nikon Eclipse 50i microscope with NIS-Elements 4.13 acquisition software (Nikon, Düsseldorf, Germany).
Flow cytometry

Lymphocyte phenotypes were determined using fluorescence-conjugated monoclonal antibodies against CD3, CD4, CD8, CD16, CD19, CD56, and TCR γδ (all from BD Pharmingen, Heidelberg, Germany). For each sample, 20,000 cells were analyzed with FACS Canto and FACS Diva Software. Intracellular FoxP3-expression was analyzed with the anti-human FoxP3 staining kit according to the manufacturers’ recommendations (eBioscience, San Diego, CA, USA). The percentage of CD25hi/FoxP3+ cells was determined after gating on CD3+/CD4+ cells.

Bone marrow chimerism

Chimerism analyses were performed as previously described using a semiquantitative PCR approach based on the amplification of short tandem repeat (STR) markers (11). Briefly, genomic DNA for multiplex PCR of microsatellite markers was extracted directly from mononuclear cells. Nine tetra-nucleotide microsatellite regions were co-amplified with dye-labeled primers using the AmpFLSTR Profiler PCR amplification kit (Applied Biosystems, Weiterstadt, Germany).
Results

Target expression in pre-therapeutic BM

TnC-A1 was expressed with a vascular and stromal pattern of staining in the BM of all three patients with BM AML involvement (Fig. 1A), providing the molecular basis for an F16-based targeting approach. In patient 1 only paraffin-embedded chloroma material was available, which could not be analyzed for TnC-A1 expression, since the F16 antibody does not work in paraffin-embedded tissues (12). However, we have previously observed that extramedullary AML lesions abundantly express TnC-A1 (5).

In vivo targeting of F16-IL2

Post-therapy BM biopsies were available in patients 2 and 4 to analyze the in vivo targeting capabilities of F16-IL2. Using an anti-IL2 antibody to detect F16-IL2 bound to its target in situ, IL2 signals decorating the extracellular matrix were detectable in the BM 6 days after the last infusion in patient 2, but not in pre-therapeutic BM (Fig. 1B), suggesting that F16-IL2 has efficiently targeted TnC-A1 in vivo and persisted for several days. On day 42, however, three weeks after the last infusion of F16-IL2, stromal IL2 signals were not observed any more (Fig. 1B, inset). In patient 4, no significant F16-IL2 signals could be observed 6 days after infusion.

Clinical courses

The most impressive response was observed in Patient 1 with disseminated extramedullary AML. Shortly after the first infusion of F16-IL2, the patient experienced pain in the cervical and the right upper abdominal chloroma lesions, suggesting a site-specific targeting of the cytokine. The palpable tumors became softer and started to shrink on the following day, and swallowing problems due to the cervical mass resolved within 48 hours while head mobility improved. All these signs of activity occurred before the beginning of additional radiotherapy, which was initiated on day 4 to ensure that the two most critical chloroma lesions (cervical mass and liver hilum) are efficiently treated (23,4 Gy). On day 14 (i.e. after 2 infusions of F16-IL2), a complete metabolic response of all irradiated and non-irradiated AML lesions could be observed in PET/CT, accompanied by a partial morphological response (Fig. 2A). While the first three infusions of F16-IL2 were well tolerated with transient fevers of two to three days after infusion, pain at sites of chloroma, and slight signs of skin and liver graft-versus-host disease (GVHD) worsening, the patient had to be hospitalized three days after the 4th
administration of the fusion protein (day 25) due to prolonged high fevers escalating to 40.5°C, respiratory distress, and increasing skin GVHD (stage 3, erythroderma). A CT scan revealed pulmonary infiltrates in the middle lobe and bilateral pleural effusions. Since clinical and laboratory findings were compatible with cytokine release associated with a macrophage activation syndrome, with elevation of ferritin levels (15,575 µg/L) on day 28, hypofibrinogenemia (81 mg/dL, 263 mg/dL prior to F16-IL2), and elevated IL6 levels (32 pg/mL, 6-fold increase from baseline), high-dose corticosteroids were administered in addition to broad-spectrum antimicrobials. The patient’s fever resolved within 48 hours, her other clinical symptoms improved within the following days, and ferritin levels decreased to 2806 µg/l within two weeks. She was discharged on day 39. The patient did not receive the planned 2nd cycle of therapy, but continued treatment with a reduced dose of 30 Mio IU of F16-IL2 in longer intervals of three weeks on an outpatient basis. She resumed normal activity with a fair quality of life (Karnofsky index of 80-90%), no clinical signs of chloroma disease, and pre-existing skin GVHD that was manageable with local and oral methylprednisolone. However, in September 2013, almost four months after the first infusion of F16-IL2, a follow-up PET/CT revealed disseminated recurrence of chloromas. Treatment was intensified to weekly applications of 50 Mio IU F16-IL2 in combination with LDAC, however, the patient did not respond a second time. Since she expressed her distinct wish for further therapy, she was offered intensive chemotherapy with sequential high-dose cytarabine and idarubicin. Unfortunately, the patient died due to infectious complications in the phase of neutropenia in November 2013 on day 176.

All other patients displayed “classical” medullary AML. Patient 2 showed a 50-60% BM infiltration before treatment. After two infusions of the immunocytokine, BM blasts as determined by cytologic analyses were reduced to 6% on day 14, and were further reduced to <5% on day 28, after four administrations of F16-IL2. Microscopic evaluation revealed therapy-induced extensive infiltration of activated lymphocytes, some with blast-like morphology in the BM during the same time. The reduction of myeloid blasts was paralleled by a disappearance of mutated NPM1 in PCR analyses and by an increase of BM donor cell chimerism from 63% to 98% (Fig. 3A). No significant leukemia infiltration was detected in flow-cytometric analyses (0.81% and 0.98% CD117+ blasts on days 14 and 28, respectively; 23.85% at baseline). Each infusion of the immunocytokine led to fever of up to 39.0°C, which typically lasted two days, but no other symptoms of cytokine release developed. Since there was no evidence of residual
leukemia at the molecular level, therapy with F16-IL2 was paused for two weeks to allow regeneration of normal hematopoiesis. By day 42, however, neutrophil and thrombocyte counts did not regenerate and AML progressed rapidly again. Treatment was resumed with higher doses of F16-IL2 (50 Mio IE on day 1, 70 Mio IE on days 8 and 15) in combination with LDAC, which, however, did not lead to a second response. The patient expressed a clear wish for further therapy and she received a third allo-HSCT three months after treatment initiation with F16-IL2. This resulted in a short CR with incomplete platelet recovery (CRp) of approximately two months, until AML relapse ultimately occurred in February 2014.

**Patient 3** presented with highly proliferative AML relapse in peripheral blood (PB) (4900 leukemic blasts/µL) and BM infiltration of 70%. Treatment with F16-IL2 (30 Mio IU) and LDAC led to a blast reduction in PB (1080/µL) within one week (Fig. 3B). However, the patient had to be hospitalized after the first dose of F16-IL2 due to fevers (39.7 °C), chills, nausea and a reduced general condition. He was treated with broad-spectrum antibacterials but it was again suspected that the fevers were more likely due to noninfectious hyperinflammation/cytokine-release. Indeed, signs of capillary leakage developed within the following days with generalized edema, weight gain (8 kg) and mild dyspnea. Laboratory findings showed elevated levels of IL6 (139 pg/mL, 10-fold increase from baseline), hyperferritinemia (11,600 µg/L) and a transient increase in creatinine (2.1 mg/dL). Fevers resolved by day 9 after four days of glucocorticoids. Creatinine normalized within four days, the patient’s weight returned to baseline by day 13, ferritin levels decreased to 3888 µg/L and he was discharged on day 15. F16-IL2 was not administered on days 8, 15 and 22. In a phase of rapid leukemia progression in PB the treatment was resumed on day 29 at 15 Mio IU F16-IL2 in an outpatient setting and was well tolerated at this time. Five days later, however, the patient had a severe accident at home and died due to intracerebral hemorrhage in the hospital shortly after.

**Patient 4** presented with at least 10% leukemic blasts (focally more) in the BM and 2% in PB. Pre-therapeutic BM donor cell chimerism decreased to 83%. Peripheral blasts disappeared within the first days of treatment with F16-IL2 and LDAC and the first BM evaluation on day 14 revealed a reduction of blasts to approximately 2% (CD33+/CD117+ cells on flow-cytometry, 12.3% at baseline) (Fig. 3C). Microscopic quantification showed ≤ 5% myeloid leukemic blasts and an extensive infiltration with activated lymphocytes. In molecular and conventional cytogenetic analyses, the markers NPM1, t(2;3) and t(5;6) were undetectable, and BM donor cell chimerism increased to 96
- 97%. Thrombocyte counts increased to 157,000/µL and neutrophil counts improved to 2214/µL after the 1st cycle (Fig. 3C), thereby meeting the criteria for a CR. However, after two cycles (two months after the first infusion of F16-IL2) the patient experienced a BM relapse with 15% blasts and reappearance of the molecular and cytogenetic abnormalities. The patient received two infusions of donor-derived lymphocytes without response and underwent a second matched unrelated alternative donor allo-HSCT in February 2014, but eventually died from infectious complications in the recovery time following transplantation.

**Toxicities**

All toxicities are summarized in Table 1. The most frequent adverse events were fever, chills, and fatigue, which occurred in all patients. IL2-related infusion-associated hypotension was not observed. Tumor pain as observed in the chloroma patient is an uncommon effect of recombinant IL2 that could be related to F16-mediated targeting of the cytokine. In patients 2 and 4, fevers typically occurred either towards the end of the infusion or shortly after, regularly resolved by day 2 or 3 and were easily manageable at home with acetaminophen. In these patients, no other relevant non-hematologic toxicities occurred (apart from mild skin GVHD in patient 2).

However, the other two patients became significantly ill after the 1st infusion of 30 Mio IU F16-IL2 (patient 3) or after the 4th infusion of 50 Mio IU F16-IL2 (patient 1), respectively. Both patients developed symptoms of the cytokine-release syndrome described above, with laboratory features compatible with macrophage activation syndrome, leading to hospitalization in both cases. The syndrome could be effectively treated with glucocorticoids. No anti-cytokine antibodies, mechanical ventilation or vasopressor support were required. Non-hematologic grade 4 events were not observed.

**Immunological effects of F16-IL2**

Circulating lymphocytes in PB significantly increased to levels 2 to 4 times as high as baseline levels by day 11 in all patients. Therapy-induced changes of peripheral lymphocyte subpopulations in patients 2 and 4 are shown in Fig. 4A. CD8+ lymphocytes peaked early after treatment initiation between days 8 and 11 with a 5.7-fold increase to 4640/µL in patient 2 and a 2-fold increase to 747/µL in patient 4. In contrast, peripheral NK cells showed a more or less steady rise with repeated administrations of F16-IL2 to values as high as 3567/µL and 1303/µL, respectively – a 17.7-fold and 16.5-fold increase.
from baseline. At peak levels, NK cells accounted for more than 50% of circulating lymphocytes. There were no significant changes in CD19+ B cells. Interestingly, activated lymphocytes and lymphocytes with large granular morphology could be readily detected in PB (Fig. 4B).

The most prominent changes were observed in the BM. F16-IL2 led to a massive accumulation of lymphocytes in the BM (Fig. 4C and D). Whereas lymphocytes accounted for 15 - 20% of cells in pre-therapeutic BM aspirates, lymphocytic infiltration of the BM was approximately 80% in patient 2 and 65% in patient 4 on day 28, coinciding with the marked reduction of leukemic blasts to molecular negativity in both patients. This phenomenon was not solely explained by the reduction of myeloid cells during therapy, but by an absolute expansion of lymphocytes. Indeed, a 10-fold increase of absolute CD8+ lymphocyte numbers, a 38-fold increase of NK cells, and a 24-fold increase of γδ T cells from baseline have been observed on day 28 in patient 4 (Fig. 4D). Comprising 45-50% of lymphocytes, NK cells represented the predominant lymphocyte fraction in the BM in both patients with maximum absolute numbers of 2798/µL and 4933/µL. In microscopy of BM aspirates, clusters of cells consisting of lymphocytes tightly attached to leukemic blasts suggestive of immunological synapse formation have been frequently observed (Fig. 4E).

T cells with regulatory phenotype (CD4+/CD25+/FoxP3+; Treg), evaluated in patient 4, accounted for approximately 3% of CD4+ T cells in peripheral and BM before treatment and expanded during therapy to 20% in both compartments on day 28.
Discussion

The increased angiogenic activity in AML BM has provided the scientific rationale for the clinical evaluation of anti-angiogenic agents in AML (8,13-15). However, therapeutic outcomes of angiogenesis inhibitors in AML have been mostly disappointing so far (16,17), stimulating the search for alternative strategies that take therapeutic advantage of angiogenic neovessels. Here, we report first-in-man experiences with the targeted delivery of IL2 to the leukemia-associated vasculature and stroma using the immunocytokine F16-IL2 in heavily pre-treated AML patients. We have chosen the situation after allo-HSCT to allow for a graft-versus-leukemia (GVL) response of a transplanted cellular immune system to the immunocytokine, and since we had previously treated two patients with late-stage refractory AML without previous allo-HSCT and had not observed significant anti-leukemic activity (data not shown).

The therapeutic principle of allo-HSCT is based on an active donor immune system that fights tumor cells of the host. We hypothesized that accumulating high doses of IL2 in the BM as the site of origin of the disease would promote GVL activity in post allo-HSCT AML patients. Indeed, immunocytokine treatment resulted in a dramatic expansion of cytotoxic effectors, which was paralleled by a striking reduction of leukemic blasts or extramedullary AML lesions. It was unexpected that only two infusions of the immunocytokine in combination with LDAC would result in a (transient) complete molecular clearance of the leukemic clone in two of three patients and in a complete metabolic response of extramedullary AML lesions as early as on day 14 after treatment initiation. The anti-leukemic effects observed with this combination therapy are highly encouraging, given that all patients were heavily pre-treated with multiple lines of intensive therapy, with two of the four patients having received even two allo-HSCTs. The observation that even patients who were previously refractory to high-dose cytarabine (3 g/m²) responded supports a substantial contribution of the targeted cytokine to the therapeutic effect. In fact, the cumulative dose of cytarabine (100 mg per cycle) was 3 to 4 times lower than the doses that are usually used in LDAC regimens (10 mg/m² or 20 mg twice daily for 10 days) (18). Furthermore, LDAC alone has not been shown to induce remissions in patients with complex karyotype AML (18).

The targeted delivery of IL2 to the BM led to a massive expansion of immune effector cells, with CD8+ T cells and NK cells representing the most up-regulated lymphocyte subsets. These observations are encouraging, in light of the fact that they
reproduce preclinical findings, which revealed a dominant role of CD8\(^+\) and NK cells for the therapeutic activity of IL2-based immunocytokines in AML-bearing mice (5).

In line with the broad immunomodulatory properties of IL2, Tregs also increased during therapy. It is entirely conceivable that Tregs might have negatively influenced the therapeutic GVL effect. Recent studies, however, which have used ultra-low doses of unconjugated IL2 to preferentially induce Tregs in an attempt to ameliorate GVHD, did not observe a diminished anti-leukemia activity or an enhanced risk of leukemia relapse after allo-HSCT (19,20). Indeed, in the context of GVHD, Tregs were shown to allow for robust GVL activity in several leukemia models (21-23). Interestingly, there is evidence that Tregs appear to not impair the immune response against tumors residing within the BM, while they readily suppress antitumor immunity if the same tumor is located outside the BM (24,25). This phenomenon has been explained by an IL1β- and IL6-mediated neutralization of the suppressive activity of Tregs by the BM stroma (in part through conversion of Tregs into IL17-producing T cells), leading to a conditional loss of Treg function in the BM and providing a favorable environment for effector cells to mediate antitumor immunity (24). Thus, it is not entirely obvious that alternative immunostimulatory cytokines which do not significantly act on Tregs (e.g., IL15) would represent better effector functions for BM-targeted immunocytokines, especially in patients with relevant GVHD. In fact, it is possible that the peripheral expansion of Tregs may have prevented even more severe manifestations of GVHD in patient 1 while still allowing for significant anti-leukemic activity of cytotoxic effectors at extramedullary sites. Combination strategies, however, which keep Treg levels low (e.g. anti-CTLA-4 antibodies) (26), might be even more efficient, yet at the expense of a potentially higher toxicity.

The fact that we observed aggravation rather than amelioration of GVHD symptoms contrasts with the notion of using IL2 to stimulate Tregs and suppress GVHD (19,20). Weekly IL2 equivalents that were administered in our patients were approximately 10 to 100-times higher than the doses used for GVHD. The difference of the dose of IL2 available in the BM is likely to be even higher, when taking into account the targeting aspect. Indeed, while CD8\(^+\) T cells and γδ T cells did not change and NK cells only doubled under treatment with low-dose IL2 (19), we have observed an up to 38-fold expansion of NK cells, a 10-fold expansion of CD8\(^+\) T cells and a 24-fold increase of γδ T cells in the BM. Thus, the local dose of IL2 equivalents is likely to
determine the net outcome of IL2-induced stimulation of pro-inflammatory and suppressive lymphocytes.

While F16-IL2 was well tolerated in two patients, the other two patients had substantial toxic side effects that either occurred immediately after the first infusion or cumulated after several infusions of F16-IL2. The cytokine-release syndrome that developed in both patients was manageable with high-dose glucocorticoids and did not reach the severity as reported for patients receiving chimeric antigen receptor-modified T cells (27). Both patients tolerated re-exposure to lower doses of F16-IL2.

In principle, it is conceivable that the observed therapeutic effects could have also partially been achieved with the administration of free, unconjugated IL2. However, there are several lines of evidence, which strongly support the contribution of the targeting aspect to the therapeutic efficacy. First, the most pronounced changes in effector cell populations have been observed in the BM as compared to PB (e.g. up to 38-fold vs. 17-fold increase in NK cell numbers in BM vs. PB, respectively), which we would not expect from a non-targeted application of IL2. The fact that we still have seen considerable immunologic effects in the PB (and systemic toxicities) could be attributed to the “fluid” nature of the BM, which is – in contrast to solid tumors – part of the immune system and dynamically connected to the PB, allowing immune effector cells to traffic between BM and the periphery and to aggravate GVHD effects. Indeed, a comparable IL2-based immunocytokine only led to a modest increase in peripheral effector cell counts in patients, in which solid tumors have been targeted (28). A dose-finding phase I study, which is currently in preparation, will tell whether lower starting doses of IL2 equivalents may lead to a more prominent discrimination between BM and PB immune effects. Second, the delivery of IL2 to the BM was demonstrated using immunohistochemical analyses of post-infusion BM biopsies. Stromal structures decorated with IL2 were detectable 6 days after the last administration of F16-IL2, indicating that the immunocytokine exhibited on-target persistence times of several days in vivo. In patients with solid tumors, comparable on-target persistence times have been described for vascular-targeting antibodies (29-33). The lack of post-therapeutic IL2 bone marrow signals in patient 4 might be explained by inter-individual differences in targeting performance in vivo. Interestingly, preliminary data suggest that angiogenesis-related matrix proteins may persist even after normalization of microvessel density in patients achieving a CR (5), potentially making TnC-A1 an attractive target for targeted-cytokines also in a post-remission setting. Third, patient 1 developed pain at
extramedullary AML sites after each of the first two infusions of the immunocytokine, a side effect that is not commonly observed with non-targeted IL2. Tumor pain upon infusion has also been observed in patients with solid tumors receiving L19-IL2, an immunocytokine that targets EDB-Fn (28,34). Last, preclinical experiments using different syngeneic and xenograft models of AML have clearly shown that complete leukemia eradication could only be achieved with vascular-targeted IL2 in combination with cytarabine, while equimolar amounts of IL2 fused to an antibody of irrelevant specificity were ineffective in inhibiting leukemia progression (5).

In summary, a combination of F16-IL2 and LDAC can provide clinically meaningful benefit to patients in the desperate situation of AML relapse after allo-HSCT. We believe that F16-IL2 combined with either LDAC or with additional immunologic maneuvers to increase on-target activity of the stimulated effector cells at the BM site, deserves further evaluation in prospective clinical trials, with a dedicated part on dose-finding, as it appears that patients with an allogeneic immune system may tolerate lower doses of F16-IL2 as compared to patients with solid tumors.

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Authorship Contributions
C.S. and W.E.B. designed the individual treatment protocols and treated the patients. A.K., M.P., S.W., G.S., L.A., T.K., R.M.M., and M.St. were involved in patient care and treatment. C.S., C.R., D.N., M.St., and W.E.B. discussed treatment results and tried to adapt and improve individual treatment protocols. L.G., K.L.G. and D.N. provided research data and F16-IL2 for compassionate use. M.Sch. performed diagnostic PET/CTs. B.A. and C.R. performed flow cytometry diagnostics. L.A. and C.S. performed immunohistochemical analyses of TnC-A1 expression. I.G., E.W., G.K., and W.E.B. performed morphologic and immunohistology diagnostics. C.S. and W.E.B. wrote the manuscript and all authors edited and approved the manuscript.
Conflict of Interest

D.N. is a co-founder and shareholder of Philogen (www.philogen.com), the biotech company which owns F16-IL2. D.N. has employment and ownership interests. All other authors declared no competing financial interest.
References


Figure Legends

Figure 1. Expression of TnC-A1 in the BM and in vivo targeting of F16-IL2. (A) Pre-therapeutic stromal expression of TnC-A1 (green) could be detected in all patients with BM involvement of AML using biotinylated F16(SIP) antibodies (200x). (B) The presence of F16-IL2 in post-therapeutic BM was highlighted using an antibody specific to human IL2 (400x). In patient 2, extracellular IL2 signals could be detected in situ 6 days (but not 3 weeks, inset) after the last infusion, suggesting successful targeting and on-target persistence of the immunocytokine for several days in vivo. Stromal IL2 signals were not detectable in pre-therapeutic BM that was not exposed to F16-IL2 treatment and used as control. (C) Schematic representation of the dimeric scFv-huIL2 fusion protein F16-IL2 in diabody format.

Figure 2. Rapid response to F16-IL2 and LDAC in disseminated extramedullary AML. (A) The figure shows axial 18-FDG-PET/CT scans obtained at baseline before treatment initiation and 14 days after the first infusion (patient 1). Pre-therapeutic images show disseminated hypermetabolic myelosarcomas (chloromas) (SUV$_{max}$ 14.1) in the left deep cervical area, in the mediastinum, in the hilum of the liver, in the mesentery, in the hilum of the right kidney, in the manubrium of the sternum, in the transverse process of the 9$^{th}$ thoracic vertebra and subcutaneously in the left gluteal area. The two clinically most critical lesions (left cervical and hilum of the liver) were irradiated between day 4 and day 24 with a cumulative dose of 23.4 Gy in addition to systemic therapy. A complete metabolic response of irradiated and all non-irradiated AML manifestations occurred within 14 days of treatment initiation, which was paralleled by a good partial morphologic remission. Panel B shows signs of skin GVHD, which occurred during therapy with F16-IL2 in this patient.

Figure 3. Clinical responses to F16-IL2 and LDAC in patients with AML BM involvement. The upper panels show PB cellular counts, specifically the development of white blood cell counts (WBC), peripheral blast counts, absolute neutrophil counts (ANC), absolute lymphocyte counts (ALC), and thrombocytes during therapy with F16-IL2 and LDAC in patients 2 (A), 3 (B) and 4 (C). Arrows indicate infusions of F16-IL2 at the dose indicated above (x 10$^6$ IU IL2 equivalents); days of application of LDAC (2 x 5 mg/day s.c.) are marked with black bars. BM blast counts as determined by flow-cytometric analyses and BM donor cell chimerism levels from aspirates taken at the time
points indicated are depicted in the lower panels (no post-therapy BM aspirates were available in patient 3).

**Figure 4. Immunologic effects of F16-IL2 therapy.** Panel (A) shows changes in PB lymphocyte subpopulations during therapy with F16-IL2 and LDAC as revealed by flow-cytometric analysis. The most prominent increase was observed in CD16+/CD56+ NK cells and CD8+ lymphocytes. Triangle-arrows indicate infusions of F16-IL2. (B) Photographs of May-Grünwald-Giemsa-stained PB smears show activated lymphocytes and large granular lymphocytes (obtained from patient 2 after two infusions of F16-IL2). (C) Immunohistochemical staining of pre- and post-therapeutic BM biopsies showed a massive influx of CD3+ and CD8+ lymphocytes as exemplified in patient 2 (CD3 staining) and patient 4 (CD8 staining). (D) Flow-cytometric analyses of BM aspirates showed a 10-fold increase of CD8+ lymphocytes and a 38-fold increase of NK cells at the end of the first cycle (patient 4). (E) Cell clusters reminiscent of immunological synapse formation between activated lymphocytes and leukemic cells could be observed frequently in the BM.
# Tables

## Table 1 Summary of adverse events

(highest grade observed according to the Common Terminology Criteria for Adverse Events, version 4.03)

<table>
<thead>
<tr>
<th>Adverse event</th>
<th>Grade</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Patient 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fever</td>
<td>2</td>
<td>Fevers up to 39.0 °C on days 1-3 after infusion of F16-IL2, manageable with oral acetaminophen in the first 3 infusions of F16-IL2</td>
</tr>
<tr>
<td>Chills</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Fatigue</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Pain</td>
<td>2</td>
<td>Pain in lymph nodes/chloroma lesions after the first two infusions of F16-IL2</td>
</tr>
<tr>
<td>GVHD</td>
<td>3</td>
<td>Significant worsening of GVHD occurred after the 3rd infusion of F16-IL2, managed with oral and topical glucocorticoids</td>
</tr>
<tr>
<td>Skin (stage)</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Liver (stage)</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Cytokine-release syndrome</td>
<td>3</td>
<td>Hospitalization on day 25 after repeated infusions of F16-IL2 due to persisting high-grade fever, respiratory distress, exacerbation of skin GVHD (erythroderma) and suspected pulmonary infection; discharge on day 39</td>
</tr>
<tr>
<td>Fever</td>
<td>3</td>
<td>Temperature escalated to 40.5 °C on day 25, resolved within two days of high-dose glucocorticoids</td>
</tr>
<tr>
<td>Hypotension</td>
<td>2</td>
<td>Only fluids, no vasopressors</td>
</tr>
<tr>
<td>Pleural effusion</td>
<td>2</td>
<td>Non-malignant pleural effusion, left thoracentesis necessary</td>
</tr>
<tr>
<td>Dyspnea</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Weight gain</td>
<td>2</td>
<td>Weight increased by 5 kg, normalized at discharge</td>
</tr>
<tr>
<td>Pulmonary infiltrates</td>
<td>3</td>
<td>Middle lobe infiltrates compatible with atypical pneumonia</td>
</tr>
<tr>
<td>Anemia</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Thrombocytopenia</td>
<td>4</td>
<td>Prolonged thrombocytopenia after the 1st cycle, resolved to grade 3 on day 73 and to grade 2 on day 84</td>
</tr>
<tr>
<td>Neutropenia</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><strong>Patient 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fever</td>
<td>2</td>
<td>Fever and chills persisted for 2 days after each infusion of F16-IL2 (peak temperature 39.5 °C), manageable with acetaminophen</td>
</tr>
<tr>
<td>Chills</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Fatigue</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>GVHD</td>
<td>1</td>
<td>Signs of mild skin GVHD developed during the 2nd cycle, resolved with topical steroids</td>
</tr>
<tr>
<td>Skin (stage)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Anemia</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Thrombocytopenia</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Neutropenia</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td><strong>Patient 3</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytokine-release syndrome</td>
<td>3</td>
<td>Signs of cytokine-release and capillary leak syndrome developed in the first days after treatment initiation,</td>
</tr>
</tbody>
</table>
hospitalization on day 1; discharge on day 15

<table>
<thead>
<tr>
<th>Symptom</th>
<th>Grade</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fever</td>
<td>2</td>
<td>Peak temperature 39.7 °C a few hours after the 1st infusion of F16-IL2, fevers persisted for one week and resolved after 4 days of glucocorticoids</td>
</tr>
<tr>
<td>Chills</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Nausea</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Elevated creatinine</td>
<td>2</td>
<td>Creatinine levels transiently increased to 2.1 mg/dL, normalization within 4 days</td>
</tr>
<tr>
<td>Weight gain</td>
<td>2</td>
<td>Increase of body weight (8 kg) due to generalized edema, normalization by day 14</td>
</tr>
<tr>
<td>Dyspnea</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Anemia</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Thrombocytopenia</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Neutropenia</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

**Patient 4**

<table>
<thead>
<tr>
<th>Symptom</th>
<th>Grade</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fever</td>
<td>2</td>
<td>Fever and chills typically occurred after the infusion of F16-IL2 (peak temperature 39.0 °C), persisted for 2-3 days after each infusion and responded well to oral paracetamol</td>
</tr>
<tr>
<td>Chills</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Fatigue</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Anemia</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Thrombocytopenia</td>
<td>3</td>
<td>Normalized on day 22</td>
</tr>
<tr>
<td>Neutropenia</td>
<td>4</td>
<td>Resolved to grade 3 on day 25 and to grade 2 on day 40</td>
</tr>
</tbody>
</table>
Fig. 1

A

Patient 2  Patient 3  Patient 4
TnC-A1  DAPI  TnC-A1  DAPI  TnC-A1  DAPI

B

Pre-therapeutic BM  Post-therapeutic BM

C

L-2  L-2
Fig. 2

A

Baseline

Day 14

B
Fig. 3

A

B

C

Bone marrow cells (%)

Donor cell chimerism

Blasts

Days

NPM1 pos.

NPM1 neg.

Days

NPM1 pos.

NPM1 neg.

NPM1 pos.

NPM1 neg.

NPM1 neg.

NPM1 pos.

NPM1 neg.

NPM1 neg.

NPM1 pos.

NPM1 neg.

NPM1 neg.

NPM1 pos.

NPM1 neg.

NPM1 neg.

NPM1 pos.
Fig. 4

A

Patient 2

Patient 4

Cells/µL

Days

Cells/µL

Days

B

C

Baseline

Day 14

CD3+

CD8+

D

Baseline

Day 28

Cells/µL

CD8+ γδ T cells CD4+ Treg NK cells CD19+

E

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Cancer Immunology Research

Targeting Interleukin-2 to the Bone Marrow Stroma for Therapy of Acute Myeloid Leukemia Relapsing After Allogeneic Hematopoietic Stem Cell Transplantation

Christoph Schliemann, Katrin L Gutbrodt, Andrea Kerkhoff, et al.

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