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CXCR4-directed Imaging and Therapy in Acute Leukemia

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Introduction

CXCR4/CXCL12 signaling, expression and function in a physiological context

C-X-C chemokine receptor 4 (CXCR4, CD184) is a G-protein coupled receptor (GPCR) that regulates a broad spectrum of physiological processes. Structurally, CXCR4 has 7 transmembrane domains and a C-terminus protruding into the cytoplasm. In concordance with the nomenclature of chemokine receptors and their ligands, the name CXCR4 is derived from the position of N-terminal cysteine residues of its ligand C-X-C chemokine 12 (CXCL12), also known as stromal cell-derived factor 1 (SDF1) (Bachelerie, Ben-Baruch et al. 2014). CXCL12 is the only chemokine-ligand of CXCR4, and binding of CXCL12 to CXCR4 leads to engagement of the G-protein α_i- and $\beta\gamma$- subunits to initiate downstream signaling events (Feng, Broder et al. 1996). The $G\alpha_i$ subunit inhibits adenylyl cyclase (AC), counteracting effects of GPCRs which primarily signal via the $G\alpha_s$ subunit. The $G\beta\gamma$-subunits activate phosphoinositide 3-kinase (PI3K) and phospholipase C-β (PLCβ), which ultimately results in Ca^{2+} release from the ER and signaling through the protein kinase B (AKT) and the mitogen-activated protein kinase (MAPK) pathways. G-protein-independent signaling induced by CXCL12 binding includes JAK-STAT phosphorylation and recruitment of β-arrestins (Vila-Coro, Rodríguez-Frade et al. 1999, Lagane, Chow et al. 2008). The binding of β-arrestins induces desensitization of cells to CXCL12 by directing CXCR4 to the lysosome and subsequently inducing its degradation (Cheng, Zhao et al. 2000).

Initially, CXCL12 was identified as a factor stimulating pre-B-cell growth (Nagasawa, Kikutani et al. 1994) before its role as a chemoattractant was discovered. It is produced by stromal cells and endothelial cells in the bone marrow, but also broadly expressed in other tissues, including brain, heart, liver and lung (Bleul, Farzan et al. 1996).

Even though CXCL12 is the only chemokine-ligand of CXCR4, other endogenous and exogenous molecules have been shown to bind to CXCR4, resulting in different biological outcomes. Migration inhibitory factor (MIF) is a cytokine with a conserved

sequence across species that plays a role in inflammatory processes, tissue regeneration and cancer. Binding of MIF to CXCR4 primarily leads to an increase in cytosolic Ca^{2+} and activation of MAPK- and AKT-signaling, which results in cellular adhesion and migration (Miller, Li et al. 2008, Klasen, Ohl et al. 2014). The essential posttranslational modifier protein ubiquitin can serve as a ligand for CXCR4 when it is secreted into the extracellular space. This interaction has been described to increase cell migration in a G-Protein-dependent manner by ultimately increasing intracellular Ca^{2+}, especially in cancer cells (Saini, Staren et al. 2011, Yan, Cai et al. 2013). The most prominent exogenous ligand which first inspired pharmacological targeting of CXCR4 is the human immunodeficiency virus (HIV)-encoded glycoprotein gp120. Together with gp41, which binds to CD4, gp120 initiates membrane fusion of HIV with its target cell by binding to either CXCR4 or chemokine receptor 5 (CCR5) (Kwong, Wyatt et al. 1998). Although inhibition of CXCR4 with the first clinically used CXCR4 inhibitor AMD3100 (plerixafor) did not lead to a clinical breakthrough in HIV therapy because concerns of cardiac toxicity and neurocognitive side-effects arose in the first trials, but orally active CXCR4 inhibitors are still being tested, with more promising initial results and toxicity profile (Hendrix, Collier et al. 2004, Stone, Dunaway et al. 2007).

CXCR4 is expressed on many cell types of the hematopoietic and immune system, including B-cells, T-cells, granulocytes, monocytes and hematopoietic stem and progenitor cells (HSPCs). A mouse model with complete CXCR4 knockout helped to elucidate the necessity of intact CXCR4-signaling in different physiological contexts. CXCR4-knockout mice are not viable and display severe developmental aberrations affecting the heart, angiogenesis, the cerebellum and hematopoiesis, particularly B-cell development and colonization of the bone marrow by immature hematopoietic cells (Zou, Kottmann et al. 1998). Importantly, CXCL12 knock-out resulted in a very similar phenotype (Nagasawa, Hirota et al. 1996), underscoring the lack of redundancy and compensatory mechanisms in the CXCL12-CXCR4 axis, at least in embryonic development. However, it is known that CXCL12 can also bind another chemokine receptor, C-X-C chemokine receptor 7 (CXCR7 or atypical chemokine receptor 3 – ACKR3), which is generally considered a decoy-receptor for CXCL12 and facilitates the generation of a CXCL12 gradient (also often referred to as “sink” for CXCL12), as evidenced by migration of neurons during embryonic development

(Memi, Abe et al. 2013). Apart from being a decoy-receptor for CXCL12, CXCR7 can homodimerize or heterodimerize with CXCR4 and initiate signaling via arrestins without recruiting G-proteins or alter signaling initiated by CXCR4 (Rajagopal, Kim et al. 2010).

One of the major functions of the CXCL12-CXCR4 in a fully developed organism is homing and retention of CXCR4-expressing cells to sites where CXCL12 is abundant (Peled, Petit et al. 1999). The fact that HSPCs are retained by the CXCL12-CXCR4 axis is further underscored by the CXCR4-inhibitor plerixafor, which efficiently mobilizes HSPCs from their bone marrow microenvironment into peripheral blood after a single dose, which led to its approval as a stem cell mobilizing agent together with granulocyte-colony stimulating factor for autologous stem cell transplantation in multiple myeloma and non-hodgkin's lymphoma (Brave, Farrell et al. 2010).

Apart from its role in leukocyte recruitment and retention in the bone marrow or sites of inflammation, angiogenesis has been shown to be influenced by CXCR4 (Salcedo, Wasserman et al. 1999, Orimo, Gupta et al. 2005). The proposed mechanism is an induction of vascular endothelial growth factor (VEGF) secretion by CXCL12-CXCR4 (Kijowski, Baj-Krzyworzeka et al. 2001).

By attracting progenitor cells from the bone marrow to sites of tissue damage, CXCR4 also regulates tissue repair and wound healing (Avniel, Arik et al. 2006, Olive, Mellad et al. 2008).

The CXCR4-axis in hematological malignancies and solid tumors

CXCR4 is frequently overexpressed cancer, including hematological malignancies, breast cancer, colorectal cancer, esophageal carcinoma, gastric carcinoma, head and neck cancer, renal cancer, lung cancer, melanoma, gynecological cancers (ovarian, vulvar, cervical), pancreatic cancer, prostate cancer, liver cancer, sarcoma and gallbladder cancer and is often associated with adverse prognosis (Zhao, Guo et al. 2015). Pathways that are activated by CXCL12 binding to CXCR4 in physiological scenarios are also known to play a role in cancer initiation and progression (Teicher and Fricker 2010, Duda, Kozin et al. 2011, Domanska, Kruizinga et al. 2013).

In solid cancers, particularly breast cancer, CXCR4 is involved in increasing metastatic potential of cancer cells, thereby increasing probability of systemic disease dissemination. It is a widely accepted theory that organs with higher CXCL12 expression are more prone to metastasis by circulating malignant cells. (Helbig, Christopherson et al. 2003, Uchida, Begum et al. 2003, Zeelenberg, Ruuls-Van Stalle et al. 2003, Mori, Doi et al. 2004). Here, I will primarily focus on the role of the CXCR4/CXCL12/CXCR7 axis in hematological malignancies due to the subject matter of the research presented.

In acute myeloid leukemia (AML) CXCR4 expression is also linked to adverse prognosis (Rombouts, Pavic et al. 2004, Spoo, Lubbert et al. 2007, Tavernier-Tardy, Cornillon et al. 2009, Bae, Oh et al. 2015). A plethora of evidence suggests that CXCR4 is upregulated during chemotherapy and mediates treatment resistance, particularly in leukemia-initiating cells that are residing in the bone marrow in close contact with stromal cells (Tavor, Petit et al. 2004, Parmar, Marz et al. 2011, Chen, Jacamo et al. 2013, Sison, McIntyre et al. 2013). Discovery of this protective role of CXCR4 for AML cells led to the idea that therapeutic targeting of CXCR4 with inhibitors like plerixafor or BL-8040 might mobilize AML cells from their protective microenvironment into the bloodstream and thereby increase efficacy of chemotherapy. Indeed, inhibiting CXCR4 leads to displacement of malignant cells and sensitizes them to chemotherapy and even fms like tyrosine kinase 3 (FLT-3) inhibitors. Direct effects of CXCR4 inhibition on AML cells include induction of cell death and inhibition of proliferation by reduction of BCL2 and MCL1 and inhibition of CXCR4 dependent ERK-phosphorylation (Zeng, Shi et al. 2009, Zhang, Patel et al. 2012, Cho, Zeng et al. 2015, Abraham, Klein et al. 2017). Interestingly, CXCR4 signaling itself can also lead to apoptosis of leukemic cells (Kremer, Peterson et al. 2013), which is attenuated by osteoblastic cells of the bone marrow niche (Kremer, Dudakovic et al. 2014). Clinical trials testing different CXCR4 inhibitors and antibodies are currently ongoing (BL-8040 Addition to Consolidation Therapy in AML Patients (BLAST): ClinicalTrials.gov Identifier: NCT02502968; CXCR4 inhibition as chemosensitizer before allogeneic stem cell transplantation: NCT02605460; CXCR4 inhibition in relapsed refractory myelodysplastic syndrome (MDS) and AML, NCT02995655).

In acute lymphoblastic leukemia (ALL) high CXCR4 expression is also associated with worse prognosis (Ko, Park et al. 2014). In B-ALL, leukemic cells show altered homing and motility characteristics (Spiegel, Kollet et al. 2004) and levels of CXCL12 are actually reduced in the bone marrow of B-ALL patients (van den Berk, van der Veer et al. 2014). Similar to AML, CXCR4 inhibition increases the potential of chemotherapeutic drugs to kill leukemic cells in their protective niches (Parameswaran, Yu et al. 2011). Recently, the CXCL12-CXCR4 axis was identified to be essential for T-ALL development and maintenance in the bone marrow microenvironment. Two elegant studies making use of conditional CXCL12 knock-out in perivascular, endothelial and osteoblastic cells and NOTCH-dependent T-ALL mouse models showed that CXCL12 and CXCR4 are essential for T-ALL disease initiation and progression (Passaro, Irigoyen et al. 2015, Pitt, Tikhonova et al. 2015). The high expression of CXCR4 on T-ALL blasts further supports its potential as a novel target molecule in T-ALL, particularly because this disease is difficult to treat and often relapses.

Apart from altered expression patterns of CXCR4 and CXCL12, mutations affecting CXCR4-activity and internalization characteristics – so called WHIM-like mutations that can be found in patients with WHIM syndrome (warts, hypogammaglobulinemia, infections, myelokathexis) – are oncogenic drivers in Waldenström macroglobulinemia (Hunter, Xu et al. 2014). In WHIM-syndrome, a very rare autosomal-dominant disease, mutations affecting C-terminal phosphorylation sites of CXCR4, which are essential for internalization and de-sensitization after binding of CXCL12, are causing constitutive activation of CXCR4 (Hernandez, Gorlin et al. 2003, Balabanian, Lagane et al. 2005). In Waldenström macroglobulinemia around 30% of patients have somatic WHIM-like mutations in CXCR4, which promote drug resistance and are associated with higher malignancy-associated immunoglobulin-M (IgM)-levels and bone marrow infiltration (Treon, Cao et al. 2014, Cao, Hunter et al. 2015, Poulain, Roumier et al. 2016).

CXCR4 mutations – both in the C-terminus similar to the ones found in WHIM syndrome, but also others with unclear functional consequences - have been reported in follicular lymphoma (FL), splenic marginal zone lymphoma (MZL) and even as one of the top genetic drivers in a large, clinically and genetically very well characterized cohort of diffuse large B-cell lymphoma (DLBCL) patients (Roccaro,

Sacco et al. 2014, Pastore, Jurinovic et al. 2015, Krysiak, Gomez et al. 2017, Reddy, Zhang et al. 2017). However, these mutations are rare and the biological and clinical significance of these *CXCR4* mutations in lymphomas apart from the well-characterized WM is still unclear.

The role of the alternative CXCL12-receptor CXCR7 in hematological malignancies is less well defined; even the question about surface expression of this receptor has caused controversy (Berahovich, Zabel et al. 2010). Data from mouse models of DLBCL suggests that CXCR7 expression might be essential for disease dissemination, especially into the central nervous system and bone marrow (Puddinu, Casella et al. 2017). In a subset of ALL patients, CXCR7 is overexpressed and collaborates with CXCR4 in inducing migration and thereby dissemination in the bone marrow (Melo, Longhini et al. 2014). In WM, CXCR7 has even been shown to be a factor increasing "stemness" of cancer-initiating cells (Wada, Ikeda et al. 2016), although more data from primary cells is needed to support this hypothesis.

CXCR4-directed positron emission tomography imaging and therapy

Given the fact that CXCR4 plays a major role in inflammation, infectious diseases and cancer biology, different imaging modalities for detection of CXCR4-expressing cells have emerged. In 2009, a method to label the CXCR4-inhibitor plerixafor with a radioactive copper isotope (^{64}Cu) enabled imaging of CXCR4-expressing cancers (Jacobson, Weiss et al. 2009). However, its use was limited by a high uptake of the compound in the liver. Preclinical testing of ^{125}I-labeled antibodies against CXCR4 showed uptake into cerebral tumor xenografts in immunocompromised mice, but this approach was not developed further (Nimmagadda, Pullambhatla et al. 2009). Another molecule that displayed favorable binding characteristics to CXCR4 and successful preclinical application is the peptide T140 and derivatives thereof (Jacobson, Weiss et al. 2010).

With the recent development of novel peptides inspired by the structure of CXCL12 that bind to CXCR4 with high affinity, the quality of CXCR4-directed imaging improved significantly. The CXCR4-binding peptide CPCR4-2 can be labeled with ^{68}Ga to produce a PET-tracer that is specific for human CXCR4 (^{68}Ga-Pentixafor) and

displayed favorable binding characteristics and imaging quality in tumor xenografts (Demmer, Dijkgraaf et al. 2011, Demmer, Gourni et al. 2011, Gourni, Demmer et al. 2011). Further clinical development of ^{68}Ga-Pentixafor showed that it has a favorable biodistribution profile with low unspecific uptake to liver or other organs (Herrmann, Lapa et al. 2015). ^{68}Ga-Pentixafor was subsequently evaluated in hematological and solid malignancies. The CXCR4-binding tracer was successfully used for imaging CXCR4 expression in lymphoma (Wester, Keller et al. 2015), multiple myeloma (Philipp-Abbrederis, Herrmann et al. 2015), pancreatic cancer, laryngeal cancer, non-small cell lung cancer, prostate cancer, melanoma, breast cancer, hepatocellular carcinoma, glioblastoma, sarcoma and cancer of unknown primary (Vag, Gerngross et al. 2016). After myocardial infarction, ^{68}Ga-Pentixafor could detect areas of inflammation in the myocardium in a mouse model and patients (Thackeray, Derlin et al. 2015).

Recently, modifications of Pentixafor allowed coupling of other radioisotopes to the peptide structure. These new CXCR4-binding peptides labeled with the beta emitters ^{90}Y or ^{177}Lu (^{90}Y- or ^{177}Lu-Pentixather) with the intention to be used as therapeutic agents to target CXCR4-expressing cancer cells and their microenvironment (Schottelius, Osl et al. 2017). Pre-therapeutic imaging for patient selection with Pentixafor and peptide-receptor radiotherapy with Pentixather is therefore considered a potential “theranostic” (therapy and diagnostics) concept. CXCR4-theranostics with Pentixafor was first applied in patients with highly treatment-resistant multiple myeloma without any further treatment options, showing effective reduction of disease burden in this patient collective (Herrmann, Schottelius et al. 2015). Importantly, CXCR4-directed peptide-receptor radiotherapy is toxic to CXCR4-expressing hematopoietic cells and therefore requires stem cell rescue.

Acute Myeloid Leukemia

AML is a rare hematological cancer with an incidence of approximately 3.7 per 100,000 individuals per year and one peak in childhood and one around the age of 65 (Deschler and Lubbert 2006). AML arises from hematopoietic stem or progenitor cells having acquired mutations or chromosomal aberrations that enable these cells

to proliferate uncontrollably. A fraction of these cancer cells is believed to have stem-cell-like properties and displays self-renewal, giving rise to the bulk of AML cells (Lapidot, Sirard et al. 1994, Bonnet and Dick 1997).

Due to progressive infiltration of the bone marrow space and the resulting impairment of healthy hematopoiesis, pancytopenia is commonly present at the time of presentation. Symptoms are often related to pancytopenia, including fatigue, infections and bleeding, all of which can be rapidly fatal if left untreated. Signs of extramedullary involvement include skin, mucosa (gingival, pharyngeal), lung infiltration (chloroma) and central nervous system involvement, although the latter is rare and not routinely tested for during initial diagnosis and the routine use of central nervous system-penetrating chemotherapeutic drugs such as cytarabine during induction treatment limits the clinical value of extensive diagnostic measures outside of clinical trials (Bakst, Tallman et al. 2011).

The most widely used risk-stratification model to estimate prognosis of AML is the European LeukemiaNet classification. In this system, patients can be grouped into favorable, intermediate-I, intermediate- II and adverse risk groups depending on molecular genetics and cytogenetics of their disease. The favorable risk group includes patients with t(8;21)(q22;q22), mutated *NPM1* without *FLT3*-ITD, mutated *CEBPA*, inv(16)(p13.1q22); the adverse group comprises complex karyotypes, del5q, t(v;11)(v;q23), t(6;9)(p23;q34), inv(3)(q21q26.2), monosomy 7, high allelic ratio of *FLT3*-ITD with non-mutated *NPM1,* mutated *ASXL1, RUNX1, TP53;* all other aberrations fall into the intermediate risk categories (Mrozek, Marcucci et al. 2012). Median overall survival rates 3 year after initial diagnosis range from 66 to 12% in younger (<60 years) patients and from 33 to 3% in older (>60 years) patients depending on the aforementioned risk groups.

Despite significant advances in the understanding of disease dynamics, clinical management of chemotherapy-related complications and biology, particularly the characterization of the genomic landscape and driver mutations in AML, the prognosis remains poor, especially for the elderly and patients unfit for dose-intense induction chemotherapy (Papaemmanuil, Gerstung et al. 2016). The standard protocol for patients fit for intense induction chemotherapy still remains cytarabine and intermittent anthracyclines (generally referred to as “7+3”). The preferred choice of anthracycline varies regionally, but mostly daunorubicin, idarubicin and

mitoxantrone are used, with daunorubicin being the most common. Consolidation treatment with high dose cytarabine after successful induction is administered to prevent relapse (Hengeveld, Suciu et al. 2012)

In older adults or patients with comorbidities or other reasons rendering them unfit for intense curative protocols, palliative treatment with azacitidine or decitabine or even best supportive care is preferred (O'Donnell, Abboud et al. 2012)

Recently, it could be shown that the addition of the Flt3 inhibitor midostaurin to standard chemotherapy improves overall survival, which lead to its approval as the first new drug in this disease in 15 years (Stone, Mandrekar et al. 2017).

One of the reasons for the dismal prognosis of AML is primary induction failure, reflecting chemoresistance, and its frequent tendency to relapse, which is partly believed to be mediated by CXCR4-induced protection of leukemic cells by the microenvironment, as mentioned above (Tsirigotis, Byrne et al. 2016). The best option for clinically fit patients with relapsed AML is dose-intense salvage chemotherapy combined with irradiation of sanctuary sites or total body irradiation (TBI) if indicated, followed by allogeneic stem cell transplantation to exploit the graft-versus-leukemia effect and kill potentially remaining leukemic stem cells and achieve durable remissions (Gillissen, Kedde et al. 2018). However, allogeneic stem cell transplantation is a high-risk procedure and can be associated with high mortality and morbidity rates. Successful therapy of relapse highly depends on response to salvage chemotherapy, but in this patient population no "gold standard" has been defined so far, making the choice of salvage regimen a highly individualized treatment decision (Grigg, Szer et al. 1999, Forman and Rowe 2013, Wattad, Weber et al. 2017).

Acute Lymphoblastic Leukemia

ALL is a group of highly aggressive diseases originating from different stages from B- or T- cell development (B- or T-ALL, respectively). If the disease presents as an accumulation of B/T-ALL blasts as a solid mass without bone marrow involvement >25%, it is classified as B/T-lymphoblastic lymphoma (LBL), but if bone marrow involvement >25% is present, the term ALL is applied irrespective of the presence of

an extramedullary bulk (Arber, Orazi et al. 2016). B-ALL can be further sub-classified depending on the expression of surface markers indicating its maturation stage by flow cytometry, i.e. pro-B-ALL (early precursor B-ALL), common ALL and late pre-B-ALL (Swerdlow, Campo et al. 2016).

The overall incidence of ALL is approximately 1.6 per 100000 persons per year, with a significant peak in childhood and adolescence, making it the most common cancer in childhood. T-ALL is rarer than B-ALL and makes up approximately one quarter of all ALL cases in adults and 15% of pediatric cases (Dores, Devesa et al. 2012).

Similar to AML, ALL patients often present with pancytopenia and the corresponding symptoms - infections, fatigue, bleeding – due to progressive bone marrow infiltration. In B-ALL, presentation as LBL is not as common as overt leukemia, but infiltration of extramedullary organs such as spleen, lymph nodes, liver, central nervous system and testes in male patients occurs frequently. T-ALL often presents with a large mediastinal tumor, generalized lymphadenopathy and central nervous system involvement. Due to the high frequency of mediastinal infiltration, complications such as superior vena cava syndrome or pleural and pericardial effusions often occur (Chiaretti, Vitale et al. 2013).

Prognosis of ALL depends on various factors, and cytogenetics, molecular genetics and clinical patient characteristics are useful for further risk estimation. Poor risk factors include Philadelphia chromosome t(9;22), leading to expression of the BCR-ABL fusion protein, *MLL* translocations, *IKZF1* mutations, T-cell immunophenotype, high leukocytes at presentation, male sex, Hispanic or black race and, very importantly, age <1 year or 10 years and older, with a further decline of prognosis in older age (Inaba, Greaves et al. 2013).

Treatment of ALL depends on age, subtype and risk constellation and regional differences in management are common. In general, the goal is to rapidly eradicate cancer cells with a combination of high doses of cytarabine, methotrexate, anthracyclins, vinca-alcaloids and Rituximab depending on the protocol (Larson, Dodge et al. 1995, Bassan and Hoelzer 2011). Central nervous system prophylaxis (intrathecal chemotherapy with or without whole brain irradiation) is applied in many protocols to prevent central nervous system relapse (Lazarus, Richards et al. 2006, Sancho, Ribera et al. 2006). Intense consolidation treatment helps preventing relapse and development of therapy-resistant subclones. In adults, allogeneic stem cell

transplantation is commonly used for fit patients with poor-risk features and generally for relapsed/refractory disease (Forman, Schmidt et al. 1991, Gokbuget, Stanze et al. 2012). Very recently, significant advances have been made in treating relapsed B-ALL with the approval of the bi-specific T-cell engager (BiTE) blinatumomab (Topp, Gokbuget et al. 2015) and the approval and standardization of CD19-specific chimeric-antigen-receptor-T-cell technology with tisagenlecleucel (Maude, Laetsch et al. 2018).

In academic centers, patients are encouraged to participate in ongoing trials of relevant study groups, for example the German Multicenter ALL Study Group (GMALL), the Berlin-Frankfurt-Münster (BFM) groups for pediatric patients and the Cancer And Leukemia Group B (CALGB), to further standardize and advance development of novel treatment options to improve outcome.

Leukemia initiating cells (LICs), also referred to as leukemic stem cells (LSCs), are important for understanding mechanisms of relapse in ALL. This cell fraction is rare and believed to be highly treatment resistant (Bernt and Armstrong 2009, Ebinger, Ozdemir et al. 2016). The recent finding that CXCR4 is relevant for LIC-function in T-ALL (Passaro, Irigoyen et al. 2015, Pitt, Tikhonova et al. 2015) argues for targeting this molecule to eradicate LICs.

Materials and methods

All methods described here can be found in the attached published manuscripts in detail (Herhaus, Habringer et al. 2016, Habringer, Lapa et al. 2018).

Cell lines and tissue culture

For in-vitro experiments we AML cell lines as follows: GF-D8, KG1a, Molm-13, Mono-Mac-1, Mono-Mac-6, MV4-11, NB4, NOMO-1, OCI-AML2, OCI-AML3, OCI-AML5. 5637 cells, a bladder cancer cell line producing multiple growth factors (Kaashoek, Mout et al. 1991), was cultured to produce a growth-factor-enriched supernatant needed for media of GF-D8 and OCI-AML5. For virus-production, HEK294T cells were used.

Roswell Park Memorial Institute medium 1640 (RPMI1640) with 20% fetal calf serum (FCS) was used to culture KG1a, Molm-13, MV4-11, NB4 and NOMO-1 cell lines.

High glucose (4.5g/L) Dulbecco's Modified Eagle Medium (DMEM) with 10% FCS was used for OCI-AML2 and OCI-AML3. RPMI with 20% FCS, 1% non-essential amino acids (NEAA) and 1mM sodium pyruvate was used for Mono-Mac-1; for Mono-Mac-6, insulin was added to a final concentration of 10μg/ml. Alpha-MEM supplemented with 20% FCS and 10% supernatant of 5637 cells was used for OCI-AML5. RPMI1640 medium with 20% FCS and 10% 5637 supernatant was used to culture GF-D8 cells.

Daudi, a Burkitt's lymphoma cell line which expresses very high levels of CXCR4 on the cell surface (Wester, Keller et al. 2015), was cultured in RPMI1640 with 20% FCS, 0.05mM β-mercaptoethanol, 1% NEAA. HEK293T cells were cultured in DMEM and 10% FCS.

To prevent contamination, all cultures were kept with 100μg/ml streptomycin and 100U/ml penicillin and tested for mycoplasma with polymerase chain reaction (PCR). Culture conditions were 37°C and 5% CO_2 for all cell lines.

All cell culture materials, media and additional factors were purchased from Life Technologies (Carlsbad, CA, USA). All cell lines mentioned above were either newly purchased from "Deutsche Sammlung von Mikroorgamismen und Zellkulturen" (DSMZ) or regularly re-tested fro authenticity.

Flow cytometry

Flow cytometry data shown in both manuscripts were either generated on a CyanADP (Beckman Coulter, Brea, CA, USA) for cultured cells, primary murine cells and patient derived xenograft cells or on a Cytomics FC500 (Beckman Coulter, Brea, CA, USA) for routine diagnostics and other patient data. Analysis of flow cytometry data was done with FlowJo (Ashland, OR, USA) or Kaluza Flow Analysis Software (Beckman Coulter, Brea, CA, USA). Antibodies used were purchased from Beckman Coulter (Brea, CA, USA), BD Biosciences (Franklin Lakes, NJ, USA) or eBioscience (San Diego, CA, USA) and are summarized in Table 1.

Table1. Antibodies used for flow cytometry.

Target	**Fluorochrome**	**Clone**	**Company**
hCD117	PE	104D2D1	Beckman Coulter
hCD13	PE	SJ1D1	Beckman Coulter
hCD14	PE	RM052	Beckman Coulter
hCD15	FITC	80H5	Beckman Coulter
hCD3	FITC	UCHT1	Beckman Coulter
hCD30	PE	HRS-4	Beckman Coulter
hCD33	PE	D3HL60.251	Beckman Coulter
hCD34	FITC	581	Beckman Coulter
hCD4	PE-Cy5	13B8.2	Beckman Coulter
hCD45	ECD	J33	Beckman Coulter
hCD45	eFluor450	HI30	eBioscience
hCD5	PE-Cy7	BC1a	Beckman Coulter
hCD56	PE	N901(NHK-1)	Beckman Coulter
hCD64	FITC	22	Beckman Coulter
hCD7	PE-Cy5	8H8.1	Beckman Coulter
hCD8	ECD	SFC/Thy2D3	Beckman Coulter
hCXCR4	PE	12G5	BD Biosciences
hCXCR4	BV421	12G5	BD Biosciences

hHLADR	ECD	Immu357	Beckman Coulter
hMPO	FITC	CLB-MPO-1	Beckman Coulter
hTdT	FITC	HT1,HT4,HT4,HT9	Beckman Coulter
Isotype ctrl	BV421	-	BD Biosciences
mB220	PE-Cy7	RA3-6B2	eBioscience
mCD117/cKit	PE	2B8	eBioscience
mCD11b	APC-eFluor780	M1/70	eBioscience
mCD166/ALCAM	PE	ALC48	eBioscience
mCD3	PE-Cy5.5	145-2C11	eBioscience
mCD31	APC	390	eBioscience
mCD4	PE-Cy5	GK1.5	eBioscience
mCD45	FITC	30F11	eBioscience
mCD45	APC-eFluor780	30F11	eBioscience
mCD8a	PE-Cy5	53-6.7	eBioscience
mGr1	PE	RB6-8C5	eBioscience
mSca1	PE-Cy7	D7	eBioscience
mTer119	eFluor450	TER-119	eBioscience
Murine lineage cocktail (CD3, B220, Gr1, CD11b, Ter119)	Biotin	-	eBioscience

Table 1. Abbreviations**:** m: murine, h: human, ECD: R Phycoerythrin-Texas Red-X, APC: Allophycocyanin, PE: Phycoerythrin, FITC: Fluorescein isothiocyanate, BV: brilliant violet.

Mice and xenograft models

For subcutaneous xenograft experiments we injected 5 million OCI-AML3, 7.5 million NOMO-1 or 6 million KG1a cells premixed with growth factor reduced matrigel (Corning Inc., Corning, NY, USA) into the flanks of immunocompromised CB17/Icr-

Prkdc^{scid}/IcrIcoCrl (SCID) mice. Tumor onset was monitored with clinical controls and measurement of tumor size.

Patient derived xenograft models (PDX) are constantly growing in popularity due to their ability to mirror the biology of human cancers in many aspects, especially in acute myeloid and lymphoid leukemia (Vick, Rothenberg et al. 2015). We chose PDX to test CXCR4-directed imaging and therapy in immunocompromised NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice, which harbor an additional mutation in the gamma chain of the interleukin 2 receptor. PDX were generated as previously described (Vick, Rothenberg et al. 2015, Ebinger, Ozdemir et al. 2016): peripheral blood or bone marrow samples of AML and ALL patients were acquired during diagnostic work-up and injected in NSG mice via tail-vein-injection. When these primary recipients developed clinically apparent symptoms of leukemic infiltration or had more than 60% of human leukemic cells in peripheral blood, mice were sacrificed, spleens and bone marrow from tibia and femur were filtered to obtain a single cell suspension and leukemic cells were enriched by centrifugation with Biocoll (Merck, Darmstadt, Germany). Then, human leukemic cells were further expanded by a secondary transplantation, spleens were again mashed and leukemic cells were frozen and stored in liquid nitrogen before being used in imaging- or treatment experiments.

All animal experiments were approved by local authorities (Regierung von Oberbayern) and conducted according to the Guide for Care and Use of Laboratory Animals as published by the United States National Institutes of Health (NIH Publication No. 85-23, revised 1996). Use of patient material was conducted in a manner that was compliant with the declaration of Helsinki, written informed consent was given by all patients before material was used and compliance with ethical standards was confirmed by the ethics committee of the LMU Munich.

Gene vectors, transfection and transduction

For gene-knock out studies in human AML cell lines, we used a CRISPR-Cas9 based approach with an all-in-one vector system (lentiCRISPR v2, Addgene plasmid #52961) coding for Streptococcus pyogenes Cas9, a single-guide RNA (sgRNA) and

a puromycin-resistance gene for selection of transduced cells (Sanjana, Shalem et al. 2014). To facilitate cloning of sgRNAs into lentiCRISPR v2, a CcdB cassette coding for the bacterial gyrase inhibitor CcdB toxin, was introduced between BsmBI restriction sites that are necessary for golden gate assembly of a given sgRNA oligonucleotide and the target vector. Thereby, growth of bacterial colonies having taken up the undigested plasmid is efficiently prevented by the CcdB toxin, which increases cloning efficiency.

To ectopically overexpress CXCR4 in AML cells, we used a lentiviral expression vector pHIV-EGFP (Addgene # 21373) containing an EF-1α promoter followed by the multiple cloning site, internal ribosomal entry site (IRES) and enhanced green fluorescent protein (EGFP) for identification of successfully transduced cells (Welm, Dijkgraaf et al. 2008). The human CXCR4 coding sequence (RefSeq NM_003467.2) was subcloned into pHIV-EGFP with sequence and ligation independent cloning (SLIC) (Jeong, Yim et al. 2012).

To produce viral particles, HEK293T cells were transfected with the VSV-G encoding plasmid pMD2.G (Addgene plasmid #12259), the packaging plasmid psPAX2 (Addgene plasmid #12259) and lentiCRISPR v2 or pHIV-eGFP/CXCR4 using Lipofectamine2000 (Thermo Fisher Scientific, Waltham, MA, USA) and cell culture medium without antibiotics for 6h, then the medium was changed to the respective target medium. After 24h, viral supernatant was removed and added to target cells. After addition of polybrene, cells were centrifuged for 1h at 32°C with 1200rpm (spin-transduction). This procedure was repeated and stably transduced cells were either selected with 2μg/ml puromycin for at least 72h or sorted for EGFP-positivity.

For validation of CXCR4 knock-out, the locus of the predicted Cas9 cutting-site in the genomic DNA of transduced cells was amplified by PCR and sequenced with conventional Sanger sequencing. A bioinformatic algorithm (tracking of indels by decomposition: TIDE) to measure gene-editing efficiency in terms of generation of insertions/deletions (indels) was applied to select appropriate sgRNAs (Brinkman, Chen et al. 2014).

Imaging and treatment of mice

CXCR4-directed PET imaging with ^{68}Ga-Pentixafor was performed with subcutaneous xenograft-bearing mice, PDX and wild-type control NSG mice. Mice were subjected to imaging when they had palpable subcutaneous tumors or human leukemic blasts were detectable in peripheral blood, in the case of PDX. Mice underwent inhalation anesthesia with isoflurane, received an injection of ^{68}Ga-labeled Pentixafor (approximately 12MBq) and images were taken after the compound was allowed to distribute for 75min. All mouse PET-images were produced on a μPET-system, analyzed and quantified on the corresponding software (Siemens, Inveon, Erlangen, Germany).
Mice were treated with ^{177}Lu-Pentixather or "cold" unlabeled Pentixather as control after 28-35 days by tail-vein injection, depending on the infiltration dynamics of the PDX or cell line. Injected specific activity ranged from 23.4 to 27.5MBq per mouse. All tail-vein injections with Pentixather were done under isoflurane anesthesia.

Real-time quantitative PCR (qPCR)

For assessment of mRNA levels of CXCR4, RNA was isolated using the RNeasy Mini Kit (Qiagen, Venlo, Netherlands). The isolated RNA was quantified with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) followed by reverse transcription to cDNA with the Omiscript Reverse Transcription Kit (Qiagen, Venlo, Netherlands). The Platinum SYBR-Green qPCR SuperMix-UDG (Thermo Fisher Scientific, Waltham, MA, USA) was used for the reaction, with ROX (Thermo Fisher Scientific, MA, USA) as a reference dye. Quantification of cDNA amplification was performed on a StepOnePlus qPCR thermocylcer (Applied Biosystems, Waltham, MA, USA) and the ΔΔCT method was used to calculate mRNA expression relative to a reference transcript (ubiquitin). Peripheral blood mononuclear cells (PBMCs) of 3 individuals without hematological or solid malignancies was used as a healthy control. Primer sequences are as follows: hCXCR4 forward: 5'-CCG TGG CAA ACT GGT ACT TT-3', reverse: 5'-TTT CAG CCA ACA GCT TCC TT-3',

hUbiquitin forward: 5'-CCT GAG GGG TGG CTG TTA AT-3', reverse: 5'-ACC ATG CAA CGA AAC CTT TAT T-3'.

Transwell migration assays

To test the capacity of human AML and ALL cells (PDX and cell lines) to migrate across a CXCL12 gradient, we plated a defined amount of cells ($2x10^5$) into the top chamber of a transwell plate with 5µm pore size (Corning Inc., Corning, NY, USA). The lower chamber was filled with appropriate medium for the respective cell type containing 100ng/ml human CXCL12 (R&D Systems, Minneapolis, MN, USA) and 0.5% bovine serum albumin (BSA) to prevent unspecific adhesion to plastic surfaces. FCS was omitted to avoid potential confounding of results by other chemokines or chemoattractants in the serum. After 4 hours of incubation at 37°C and 5%CO_2 the amount of cells which successfully migrated across the membrane was quantified with CountBright absolute counting beads (Thermo Fisher, Waltham, MA, USA) according to the manufacturer's protocol on a CyanADP flow cytometer.

Colony-forming-unit assays and co-culture

To assess the potential of murine hematopoietic progenitor cells to form colonies we performed colony forming unit (CFU) assays. Murine bone marrow cells were plated in methyl-cellulose with appropriate murine growth factors M3434 (Stemcell Technologies, Vancouver, Canada) according to the manufacturer's protocol. Human bone marrow cells were enriched for CD34 positivity using CD34-MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and then mixed with StemMACS HSC-CFU complete with EPO (Miltenyi Biotec, Bergisch Gladbach, Germany) and plated according to the manufacturer's protocol. Colonies were categorized as GEMM (granulocyte, erythrocyte, monocyte, megakaryocyte), GM (granulocyte, monocyte), G (granulocyte), M (monocyte) or BFU-E (burst forming unit, erythrocyte). Both murine and human mesenchymal stem cells (MSCs) were isolated based on their ability to adhere to plastic surfaces, in this case cell culture flasks (Oostendorp,

Harvey et al. 2002). Murine MSCs were cultured in Alpha MEM plus Glutamax with 10% FCS, 0.1% Beta Mercaptoethanol, 100μg/ml streptomycin and 100U/ml penicillin.

Human MSCs from donors without hematological malignancies were kept in low-glucose (1g/L) DMEM, 10% pooled human platelet lysate, Heparin (50U/mL), 2mM L-Glutamine, 100μg/ml streptomycin and 100U/ml penicillin.

For co-culture of HSPCs and MSCs, murine bone marrow cells were lineage-depleted with magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) to enrich for HSPCs and plated on a MSC layer. Human HSPCs were isolated using CD34-MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and plated on human MSCs for *in-vitro* therapy experiments.

Imaging and treatment of patients

The radiopharmaceuticals Pentixather and Pentixafor were synthesized and labeled in a good-manufacturing-practice (GMP) compliant manner in a system developed by SCINTOMICS GmbH (Fürstenfeldbruck, Germany) as previously described (Demmer, Gourni et al. 2011, Bodei, Mueller-Brand et al. 2013). Quality controls of the final product were performed before injection.

All patients who were treated with Pentixather had active, relapsed AML after first allogeneic stem cell transplantation without any approved treatment options available.

For CXCR4-directed imaging, 161-340MBq of ^{68}Ga-Pentixafor was administered and scans were taken on a Siemens Biograph mMR PET/MRI (Siemens Healthineers, Erlangen, Germany). Patients were asked to fast 4 hours before imaging with ^{68}Ga-Pentixafor.

To estimate potential off-target toxicities, bone marrow dose and pharmacokinetic profile, low-dose (approximately 200MBq) ^{177}Lu-Pentixather was given and single-photon emission computed tomography (SPECT) based dosimetry studies were done.

Depending on dosimetry results, appropriate doses of ^{90}Y-Pentixather were administered on day 4 and day 7 after dosimetry under close monitoring of vital

signs. As recommended for PRRT in neuroendocrine tumors, 2L of arginine and lysine (25g/L) were given for renal protection. ^{90}Y-Pentixather was followed by conventional conditioning with chemotherapeutic drugs, and ^{153}Sm-EDTMP or ^{188}Re-anti-CD66 to increase bone marrow dose.
Patients were extensively informed about risks and potential toxicities of the given radiopharmaceuticals and gave written informed consent before imaging or therapy. Experimental treatments and imaging studies were given in concordance with the German Drug Act (§13,2b; compassionate use) and the Declaration of Helsinki (§37). Additionally, the local ethics committee approved treatment and imaging procedures.

Patients

CXCR4 surface expression was assessed by flow cytometry in 67 patients treated at our institution for AML, secondary AML or myelodysplastic syndrome (MDS).
Further 10 patients with AML (not included in the 67 aforementioned patients) underwent ^{68}Ga-Pentixafor imaging, with 5 patients with solid tumors as controls for unspecific uptake in the bone marrow.
Three patients with AML underwent CXCR4-directed PRRT and second allogeneic stem cell transplantation.

Statistics

For statistical analysis of data presented here, GraphPad Prism (GraphPad Software, La Jolla, CA, USA) was used. To assess statistical significance, we performed unpaired Student's T-tests. Differences were considered significant for p values lower than 0.05 and were displayed as * for $p<0.05$, ** for $p<0.01$ and *** for $p<0.001$ in the figures presented in both publications. To determine significant correlation, the Pearson correlation coefficient r was used. The error bars shown either display standard deviation (SD) or standard error of the mean (SEM), as indicated in the corresponding figure legend.

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Targeted positron emission tomography imaging of CXCR4 expression in patients with acute myeloid leukemia

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ABSTRACT

Acute myeloid leukemia originates from leukemia-initiating cells that reside in the protective bone marrow niche. CXCR4/CXCL12 interaction is crucially involved in recruitment and retention of leukemia-initiating cells within this niche. Various drugs targeting this pathway have entered clinical trials. To evaluate CXCR4 imaging in acute myeloid leukemia, we first tested CXCR4 expression in patient-derived primary blasts. Flow cytometry revealed that high blast counts in patients with acute myeloid leukemia correlate with high CXCR4 expression. The wide range of CXCR4 surface expression in patients was reflected in cell lines of acute myeloid leukemia. Next, we evaluated the CXCR4-specific peptide Pentixafor by positron emission tomography imaging in mice harboring CXCR4 positive and CXCR4 negative leukemia xenografts, and in 10 patients with active disease. [^{68}Ga]Pentixafor-positron emission tomography showed specific measurable disease in murine CXCR4 positive xenografts, but not when CXCR4 was knocked out with CRISPR/Cas9 gene editing. Five of 10 patients showed tracer uptake correlating well with leukemia infiltration assessed by magnetic resonance imaging. The mean maximal standard uptake value was significantly higher in visually CXCR4 positive patients compared to CXCR4 negative patients. In summary, *in vivo* molecular CXCR4 imaging by means of positron emission tomography is feasible in acute myeloid leukemia. These data provide a framework for future diagnostic and theranostic approaches targeting the CXCR4/CXCL12-defined leukemia-initiating cell niche.

Introduction

Acute myeloid leukemia (AML) is an aggressive hematologic neoplasm originating from a myeloid hematopoietic stem/precursor cell (HSPC). AML is rapidly fatal if untreated. Although rates of complete remission after initial induction chemotherapy approach 70%, many patients relapse. Prognosis remains particularly dismal for those patients with adverse prognostic disease features (i.e. poor risk cytogenetics and/or poor risk molecular genetics), as well as for elderly patients unable to undergo intensive therapy, highlighting the clinical need for effective novel therapeutic strategies.[1-3]

Acute myeloid leukemia relapses are thought to arise from quiescent leukemia-

initiating cells (LIC) harbored by the specialized bone marrow (BM) microenvironment, termed the stem cell niche. Several pre-clinical studies have shown that LICs are resistant to conventional chemotherapy as well as targeted therapy, and are selectively protected by interaction with the stem cell niche. Cross-talk between LICs and niche cells has also been demonstrated to be important for disease maintenance and progression.[4-6] Thus, targeting the BM niche is an emerging and attractive therapeutic concept in AML.

CXC-motif chemokine receptor 4 (CXCR4) functions together with its sole known chemokine ligand CXCL12 (also named Stromal cell-derived factor-1, SDF-1) as a master regulator of leukocyte migration and homing, and of HSPC retention in BM niches.[7-11] CXCR4 is physiologically expressed on myeloid and lymphoid cells as well as on subtypes of epithelial cells. The activation of the CXCR4/CXCL12 pathway has been identified in several hematologic and solid malignancies.[12] In this context, the CXCR4/CXCL12 axis is a key regulator of proliferation, chemotaxis to organs that secrete CXCL12, and aberrant angiogenesis, all of which are pivotal mechanisms of tumor progression and metastasis.[13] The interaction between CXCR4 on malignant cells and secreted CXCL12 from the microenvironment is a fundamental component of the crosstalk between LIC and their niche.[14] The CXCR4/CXCL12 axis is essential for both normal and leukemic HSPC migration *in vivo*.[15,16] In NOD/SCID mice, homing and subsequent engraftment of normal human or AML HSPC are dependent on the expression of cell surface CXCR4, and CXCL12 produced within the murine BM niche.[9,14]

As shown for several other cancers, CXCR4 expression negatively impacts prognosis in AML.[17] Recent data in acute lymphoblastic leukemia (ALL) further substantiate the crucial role of this interaction in acute leukemia.[18,19] Therefore, targeting CXCR4 and the CXCR4/CXCL12-defined LIC niche is an obvious and highly promising approach for long-term cure of hematopoietic stem cell malignancies, and CXCR4 is clearly a druggable target. Consequently, several novel therapies involving antibodies or small molecule drugs directed against CXCR4 or CXCL12 are currently being evaluated in clinical trials, with encouraging results.[20-22]

Our previous work identified the high affinity/specificity CXCR4-binding peptide Pentixafor as a suitable tracer for molecular *in vivo* CXCR4 positron emission tomography (PET) imaging in lymphoid malignancies.[23,24] Beyond imaging, however, and in particular in systemic malignancies like lymphoma and leukemia, the real impact of such a peptide would be its therapeutic application. Pentixafor labeled to therapeutic radionuclides is feasible and has already been applied in individual patients with multiple myeloma,[25] and a phase I/II clinical trial is currently under investigation (*EudraCT: 2015-001817-28*). The data presented here identify CXCR4 as a suitable target for imaging in AML, implying the potential for CXCR4-directed peptide-receptor radiotherapy (PRRT) in acute leukemia.

Methods

Patients

Samples from 67 unselected patients with active myeloid disease (myelodysplastic syndrome (MDS), *de novo* AML or secondary AML (sAML) were investigated for CXCR4 surface expression by flow cytometry.

Ten patients with active myeloid disease underwent PET imaging for CXCR4. Five patients with non-hematologic malignancies examined through different analytical approaches served as controls. As previously reported for other [^{68}Ga]-labeled peptides,[26] [^{68}Ga]Pentixafor was administered under the conditions of pharmaceutical law (The German Medicinal Products Act, AMG, Section 13, 2b) according to the German law and in accordance with the regulatory agencies responsible (Regierung von Oberbayern). All patients gave written informed consent prior to the investigation. The Ethics Committee of the Technische Universität München approved data analysis. Detailed information on patients' characteristics are provided in the *Online Supplementary Appendix*.

Cell lines

The following human AML cell lines were used: Molm-13, MV4-11, NOMO-1, NB4, KG1a, OCI-AML2, OCI-AML3, Mono-Mac-1, Mono-Mac-6, OCI-AML5, GF-D8. The human Burkitt lymphoma line Daudi served as a positive control for CXCR4 expression. For details see the *Online Supplementary Appendix.*

RNA isolation and real-time PCR

Assessment of CXCR4 mRNA of AML cell lines was performed as described in the *Online Supplementary Appendix.*

CRISPR-Cas9 mediated knock-out of CXCR4

OCI-AML3 cells were stably transduced with lentiCRISPRv2 (Addgene plasmid #52961), coding for Cas9 and a CXCR4-specific sgRNA. Indel formation was assessed as described previously.[27] Additional information is provided in the *Online Supplementary Appendix.*

Migration assay

Cell migration towards CXCL12 (R&D Systems, Minneapolis, MN, USA) was performed in transwell plates with 5 μm pore size (Corning Inc., Corning, NY, USA) and was quantified with CountBright beads (Thermo Fisher, Waltham, MA, USA). For details see the *Online Supplementary Appendix*.

Mice and tumor xenograft experiments

Animal studies were performed in agreement with the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication n. 85-23, revised 1996), in compliance with the German law on the protection of animals, and with the approval of the regional authorities responsible (Regierung von Oberbayern). PET scans of xenotransplanted AML cell lines in SCID mice were performed as previously described[24] and are described briefly in the *Online Supplementary Appendix.*

Flow cytometry and immunohistochemistry

The following antibodies were used for flow cytometry: Beckman Coulter: CD45-ECD (clone J33), CD34-FITC (clone 581), CD117-PE (clone 104D2D1); BD Biosciences (Franklin Lakes, NJ, USA): CXCR4-PE (clone 12G5), PE mouse IgG2a (Clone G155-178); for immunohistochemistry: ab12482 (clone UMB-2, abcam, Cambridge, UK), CD34 (QBEnd/10, Cell Marque), CD117 (c-kit, Dako), CD43 (Novocastra). Further details are provided in the *Online Supplementary Appendix.*

PET/MR and PET/CT imaging studies in patients and animals

[^{68}Ga]Pentixafor was synthesized and PET/MRI analysis was performed as previously described.[28-33] Detailed descriptions of

imaging protocols are provided in the *Online Supplementary Appendix*.

Statistical analysis

All statistical tests were performed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). $P<0.05$ was considered statistically significant. Quantitative values were expressed as mean±standard deviation (SD) or standard error of the mean (SEM) as indicated. Additional information is given in the *Online Supplementary Appendix*.

Results

CXCR4 is highly expressed on leukemic blasts in a subset of AML patients

To address CXCR4 abundance in myeloid malignancies, we first assessed CXCR4 expression in an unselected cohort of 67 consecutive patients with active disease (AML, MDS) by flow cytometry of bone marrow (BM) and/or peripheral blood (PB). For details of patients' characteristics see *Online Supplementary Table S1*. Myeloid blasts were gated as $CD45^{dim}$ cells, and CD117 was used as a marker for myeloid blasts (gating strategy depicted in *Online Supplementary Figure S1A*). Lymphocytes with known CXCR4 positivity served as an intraindividual control (*Online Supplementary Figure S1B*). These analyses revealed a wide range of surface CXCR4 expression on myeloid blasts, from virtually absent expression to high levels in a distinct subset of AML patients. Representative flow cytometry data from AML patients are shown in Figure 1A. Quantification of CXCR4 surface expression showed significantly higher CXCR4 expression in patients with a blast percentage exceeding 30%. There was a trend towards higher CXCR4 expression in blasts derived from AML samples compared to MDS samples (Figure 1B and C). No significant correlation between high CXCR4 expression on blasts and disease stage (first diagnosis *vs.* refractory/relapsed disease), *de novo vs.* sAML, age (<65 *vs.* ≥65 years), prognostic risk group according to the modified ELN classification[34] or existing genetic aberrations was found (*Online Supplementary Figure S2A-F*). No significantly different CXCR4 surface expression in paired PB and BM samples was observed (*Online Supplementary Figure S2G*).

[^{68}Ga]Pentixafor-PET enables in vivo CXCR4 imaging of AML xenografts

Since CXCR4 is an attractive target for novel therapeutic approaches directed against the leukemic microenvironment, we sought to evaluate the clinical applicability of the novel CXCR4-binding PET tracer Pentixafor labeled with a Gallium isotope (^{68}Ga), [^{68}Ga]Pentixafor, in myeloid malignancies. To select appropriate AML cell lines to model AML with detectable CXCR4 expression, transcript levels and surface expression of CXCR4 was evaluated in ten established AML cell lines. As expected from flow cytometry data in AML patients (Figure 1), CXCR4 expression in cell lines ranged from low (KG1a) to high (NOMO-1, OCI-AML3) (Figure 2A and B). CXCR4 surface expression assessed by flow cytometry correlated with transcript levels (Figure 2C and D). Of all cell lines analyzed, OCI-AML3 showed the highest expression and was, therefore, chosen as a cell line for modeling CXCR4-high AML in further imaging experiments.

To test if PET imaging of AML cells with [^{68}Ga]Pentixafor was feasible *in vivo*, we chose OCI-AML3 and NOMO-1 as $CXCR4^{high}$ and KG1a as $CXCR4^{low}$ cell line to generate subcutaneous xenograft mouse models. After tumor engraftment was apparent in all mice, [^{68}Ga]Pentixafor and PET imaging was performed. NOMO-1 and OCI-AML3 xenografts were clearly visible, whereas KG1a xenografts were not (Figure 3A), demonstrating that CXCR4-high AML cells can be visualized with [^{68}Ga]Pentixafor *in vivo*.

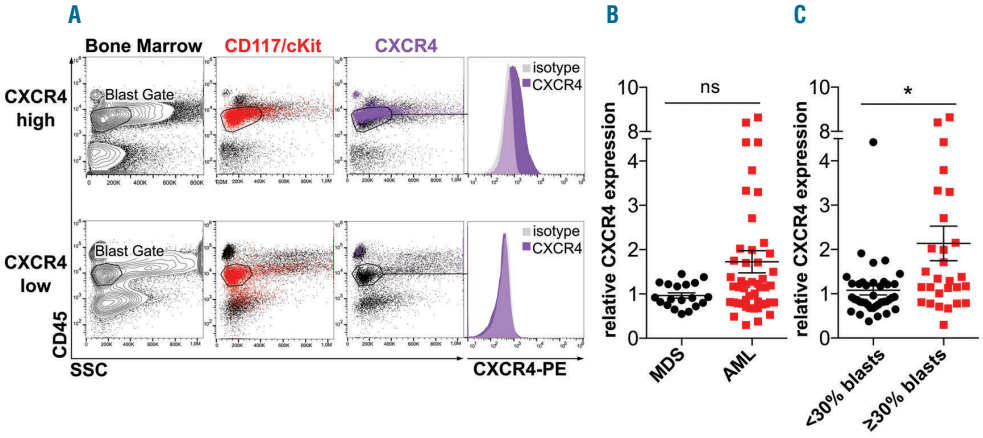

Figure 1. CXCR4 expression in patients with acute myeloid leukemia (AML) and myelodysplastic syndromes (MDS). (A) Flow cytometric evaluation of CXCR4 surface expression using an anti-CXCR4 antibody. Blasts were gated as $CD45^{low}$ cell population. Anti-CD117 antibody was used for back-gating. Representative data of CXCR4 positive (upper panels) and CXCR4 negative (lower panels) patients are shown. (B and C) Median fluorescence intensity of surface CXCR4 expression relative to isotype control (n=67 patients). Horizontal bars indicate the mean of all individual patient values±SEM; Student's *t*-test was used to compare mean relative blast CXCR4 expression. *Statistically significant differences between the groups. (B) MDS *versus* AML; $P=0.062$. (C) CXCR4 expression in patients with less than 30% blasts *versus* CXCR4 expression in patients with at least 30% blasts; $P=0.004$.

To further test the specificity of [^{68}Ga]Pentixafor binding to CXCR4, OCI-AML3 cells were selected for a CRISPR-Cas9 based stable knock-out of CXCR4 using a modified lentiCRISPRv2 to co-express *Streptococcus pyogenes* Cas9 and sgRNAs directed against human CXCR4.[35] This approach resulted in effective indel formation in the CXCR4 gene (*Online Supplementary Figure S3A*), reduction of CXCR4 surface expression (Figure 3B) and CXCL12-dependent migration (Figure 3C), while the growth kinetics remained unaffected *in vivo* (Figure 3D) and *in vitro* (*Online Supplementary Figure S3C*). For *in vivo* experiments, sg2 (sequence in *Online Supplementary Figure S3A*), targeting exon 2 of CXCR4, was chosen. OCI-AML3 stably transduced with lentiCRISPRv2-sg2 and non-targeting lentiCRISPRv2 as control were subcutaneously injected into SCID mice. [^{68}Ga]Pentixafor-PET imaging of these AML xenografts showed that OCI-AML3 control cells could be detected, and knock-out of CXCR4 in the same cell line abolished binding and PET positivity of AML xenografts. Binding of the imaging probe to mouse tissues was low, owing to the known specificity of [^{68}Ga]Pentixafor to human CXCR4 (Figure 3B and E).

Thus, *in vivo* PET imaging of AML xenografts with [^{68}Ga]Pentixafor is feasible and enables visualizing AML cells in a CXCR4-dependent manner.

CXCR4 directed PET/MR imaging in patients with myeloid malignancies

Our findings in the AML xenograft model (Figure 3), the specific binding characteristics of [^{68}Ga]Pentixafor to human CXCR4,[23,24] as well as the expression data generated in the flow cytometry patient cohort (Figure 1) encouraged us to test if CXCR4 imaging was also feasible in patients with myeloid malignancies. For this purpose, CXCR4-directed PET was combined with MR imaging, a method that is suitable for evaluating replacement of normal BM by malignant processes, including AML.[36]

Ten patients underwent [^{68}Ga]Pentixafor-PET imaging after signing informed consent. In 9 of the 10 patients, PET was combined with a whole body magnetic resonance (MR) imaging approach. In one patient, a PET/CT was conducted. One patient with extramedullary relapse and absence of BM infiltration as shown by biopsy received [^{68}Ga]Pentixafor-PET/MR and standard [^{18}F]FDG-PET/CT. Eight of 10 patients who underwent PET/MR imaging had BM involvement of AML, and one had an MDS-RAEB2. For details of patients' characteristics see *Online Supplementary Table S3*.

Four out of 9 patients with BM involvement were visually positive as assessed by [^{68}Ga]Pentixafor-PET. The PET positive areas correlated well with the expected signal

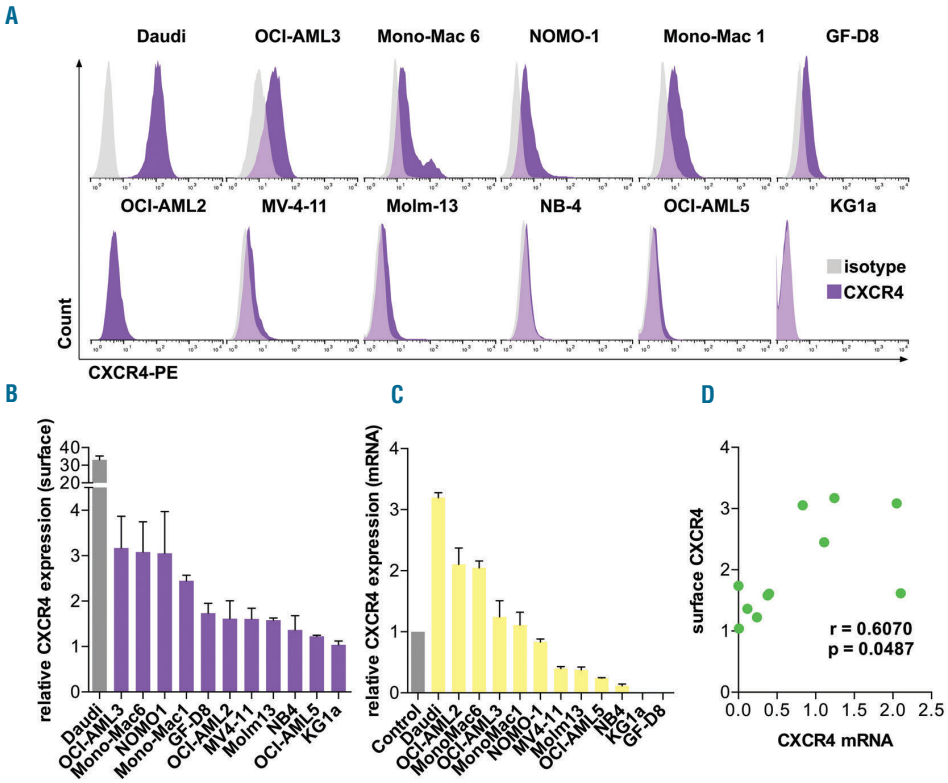

Figure 2. Surface CXCR4 expression of acute myeloid leukemia (AML) cell lines correlates with transcript levels. (A) Flow cytometric evaluation of CXCR4 surface expression of the indicated AML cell lines using an anti-CXCR4 antibody. An isotype control antibody was used as a control. (B) Mean fluorescence intensity of surface CXCR4 expression relative to isotype control. Three replicates for each cell line were used. (C) CXCR4 transcript levels measured by qRT-PCR. Mean relative expression±SEM is shown (n=3 independent experiments). ΔΔCt values relative to ubiquitin (Ub) were normalized to those of peripheral blood mononuclear cells (PBMC) of 3 healthy individuals. (D) Correlation analysis between relative CXCR4 transcript and relative CXCR4 surface expression levels.

alterations as determined by MR imaging (n=4, representative images shown in Figure 4A-F). Five of the 9 patients were visually graded as PET negative (representative images shown in Figure 4G-I). To clearly depict those differences between PET positive and negative AML and control patients, the vertebra are the best examples. Whereas all AML patients show decreased BM signal in the T1w MR sequences (Figure 4B, E, H), those BM areas only show elevated tracer uptake in the PET positive patients (Figure 4C and F). The tracer uptake within the infiltrated BM areas of the PET negative AML patient (Figure 4I) resembles those of the control patient without BM signal alterations in T1w MR sequences (Figure 4K and L). In order to allow for standardized evaluation of SUV, 5 anatomic locations with active hematopoiesis in adults were chosen for the quantification of the PET signal (Figure 4M). Compared to visually PET negative AML patients and patients with non-hematologic malignancies, the SUVmax of the five pre-defined areas of measurement was significantly higher in PET positive patients (Figure 4M). The calculated meanSUVmax was significantly higher in patients with PET positive AML compared to PET negative AML (Figure 4N). One of the 10 patients imaged with [^{68}Ga]Pentixafor-PET had biopsy-proven extramedullary relapse of AML after allogeneic stem cell transplantation (SCT) in the absence of BM involvement. [^{68}Ga]Pentixafor-PET/CT imaging in this patient revealed visually positive extramedullary disease and normal background BM signal. The extramedullary lesion showed a SUVmax of 5.2, comparable to the meanSUVmax measured in the BM of [^{68}Ga]Pentixafor-PET positive patients. Moreover, this CXCR4 positive lesion displayed high [^{18}F]FDG uptake (SUVmax 9.51) in the routine diagnostic [^{18}F]FDG-PET (*Online Supplementary Figure S4*).

To correlate *in vivo* imaging of CXCR4 with its expression level within the AML compartment, immunohistochemistry for CXCR4 was performed in 3 patients where BM biopsies in close time proximity to PET imaging were available. The high CXCR4 expression determined by IHC in Patient #1 and Patient #4 correlated well with tracer uptake detected by [^{68}Ga]Pentixafor-PET. Patient #10, who was visually negative in [^{68}Ga]Pentixafor-PET, revealed an undetectable to low CXCR4 expression as assessed by IHC (Figure 5).

In summary, these results reveal that *in vivo* imaging of myeloid malignancies, especially AML, is feasible with the new PET-tracer [^{68}Ga]Pentixafor. The variability in PET positivity for CXCR4 reflects the wide range of CXCR4 surface expression obtained with flow cytometry. Due to the limited number of patients, and the missing data on CXCR4 surface expression at the time of imaging in several patients, a statistically significant correlation between Pentixafor uptake and CXCR4 surface expression analyzed by flow cytometry and/or IHC cannot be made at this time; this will be investigated in a large planned prospective study (*EudraCT 2014-003411-12*).

Discussion

There are compelling data to show that the BM microenvironment contributes to treatment resistance and relapse in AML. CXCR4 and its ligand CXCL12 are essential for retention of normal HSPC and LICs within their protective niche and are, therefore, considered attractive targets for overcoming microenvironment-mediated resistance and inevitable subsequent clinical leukemia relapse.

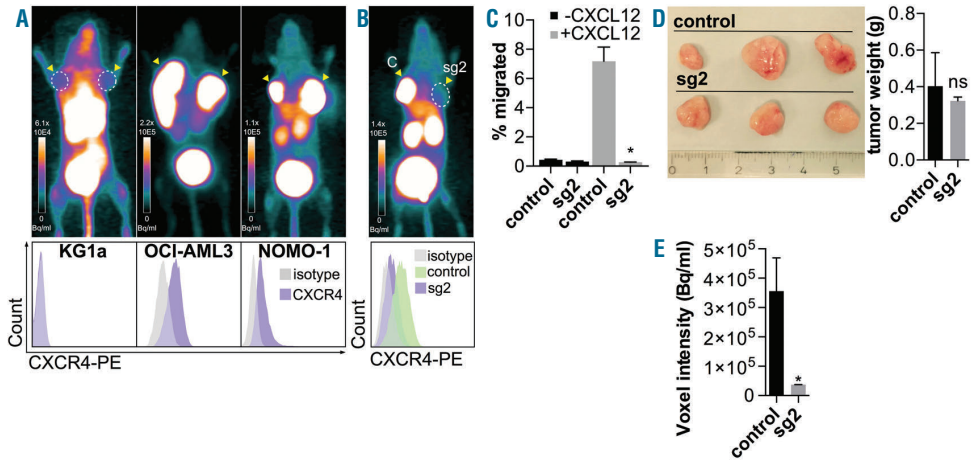

Figure 3. *In vivo* Pentixafor PET imaging in acute myeloid leukemia (AML) correlates with CXCR4 surface expression and migration towards a CXCL12 gradient. (A) [^{68}Ga]Pentixafor-PET imaging of AML xenografts. The indicated cell lines were injected into immunodeficient mice to generate xenograft tumors. CXCR4 expression was then analyzed using *in vivo* [^{68}Ga]Pentixafor-PET (upper panels). CXCR4 surface expression was analyzed by flow cytometry (lower panels). N=2 tumors/cell line; n=1 mouse/cell line. (B) [^{68}Ga]Pentixafor-PET imaging of control and CXCR4 knock-out (sg2) OCI-AML3 xenografts (upper panel). The lower panel shows CXCR4 surface expression as assessed by CD184 flow cytometry. A representative image and histogram is shown. (C) CRISPR/Cas9-mediated CXCR4 knock-out results in significantly reduced migration towards a CXCL12 gradient. OCI-AML3 cells were assessed using a transwell chamber migration assay. N=3 independent experiments. Mean±SEM is shown. *P=0.002 (Student's t-test). (D) Images of the explanted tumor shown in (B) and (C) (left panel). Tumor weight (right panel). Mean±SEM, no significant difference. (E) Quantification of [68Ga]Pentixafor uptake. Xenograft tumors were analyzed by means of voxel intensity measurement. Mean±SEM is shown, n=3 tumors for control and sg2, n=3 mice; *P=0.049 (Student's t-test).

The clinical significance of CXCR4 in AML is underscored by data showing that high CXCR4 expression on AML blasts correlates with poor prognosis.[17,37-39] In a pediatric AML cohort, blast CXCR4 surface expression was increased by chemotherapy and contributed to resistance.[40] There was no significant difference in CXCR4 surface expression between prognostic groups according to the modified ELN prognostic system[34] in our cohort, possibly due to sample size. In agreement with previous studies, CXCR4 surface expression in our cohort was highly variable. High CXCR4 expression correlated with high blast counts in our cohort, which might account for the poor prognosis seen in other studies. In addition to aberrant expression of CXCR4 in a substantial proportion of AML patients, ligand-mediated phosphorylation of serine 339 of CXCR4 appears to drive resistance to chemotherapy, and to increase retention of AML cells within the BM.[41] Such augmented interaction with the BM niche, in partic-

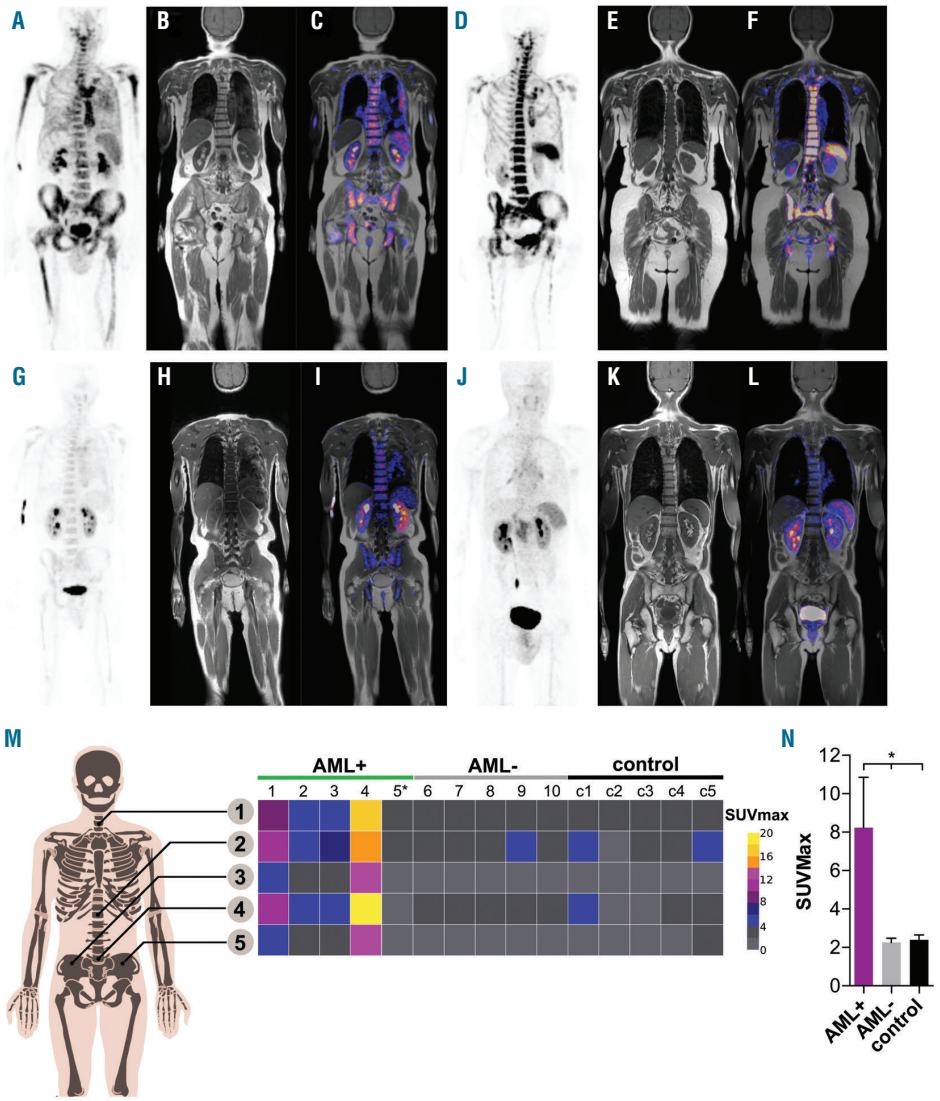

Figure 4. [^{68}Ga]Pentixafor-PET/magnetic resonance (MR) imaging in acute myeloid leukemia (AML) patients. (A-F) Shown are 2 AML patients (#2 and #1) with visually positive [^{68}Ga]Pentixafor-PET/MR imaging. (G-I)[^{68}Ga]Pentixafor-PET/MR images of a visually negative AML patient. (J-L) Control patient without BM malignancy who underwent [^{68}Ga]Pentixafor-PET/MR imaging. (A, D, G, J) Maximum intensity projections of [^{68}Ga]Pentixafor uptake. (B, E, H, K) T1w MR imaging coronal sections. (C, F, I, L) Coronal PET/MR imaging fusion. (M) (Left) Schematic graph of locations assessed for SUV quantification. 1: cervical vertebra (7); 2: thoracic vertebra (12); 3: right os ilium; 4: lumbal vertebra (5); 5: left os ilium. (Right) Heatmap of SUV values in the 5 visually positive (AML$^+$), 5 visually negative (AML$^-$), and 5 control patients with non-hematologic disease (control). *Patient #5 was scored positive because of a [^{68}Ga]Pentixafor-PET positive extramedullary lesion. (N) Quantification of SUV values from (m). *P=0.036 for AML$^+$ *versus* AML$^-$ and P=0.040 for AML$^+$ *versus* control. Error Bars represent the SEM. Patient #5 was excluded due to the lack of bone marrow involvement (extramedullary AML).

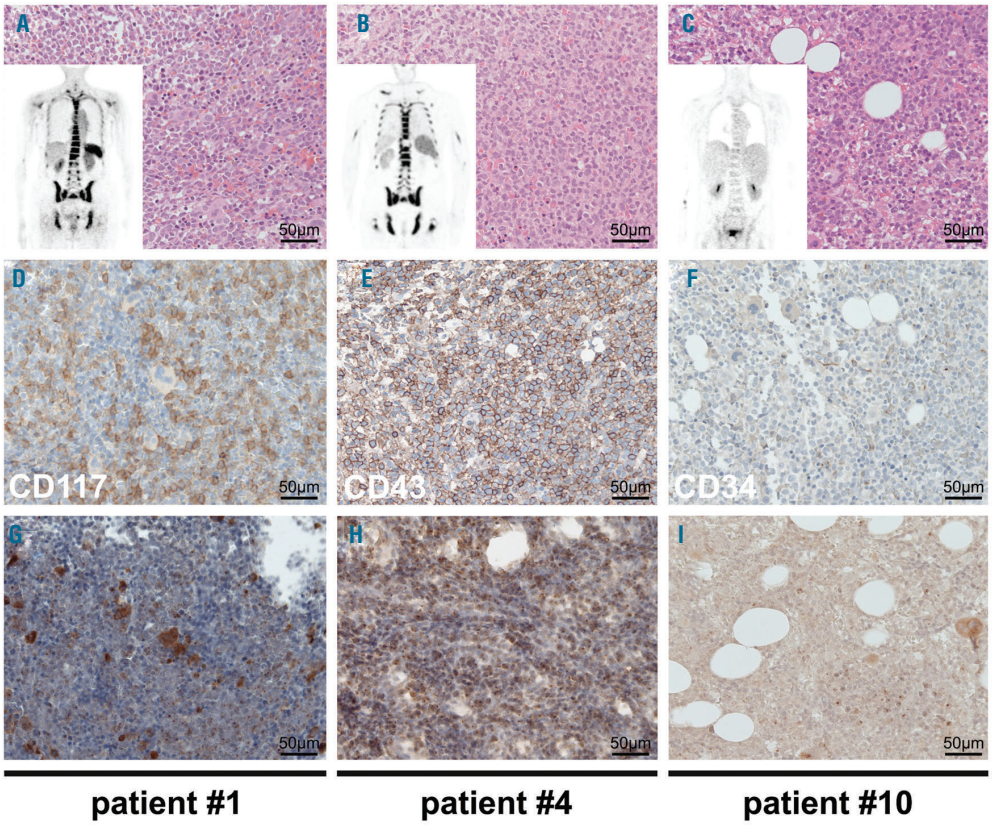

Figure 5. CXCR4 expression in bone marrow of acute myeloid leukemia (AML) patients undergoing [^{68}Ga]Pentixafor imaging. (A-C) Representative H&E stains of 3 AML patients show hypercellular bone marrow (BM) with blast infiltration; embedded are the PET images of the corresponding patients; (A) and (B) are visually positive for CXCR4-directed PET and (C) is negative. (D-F) IHC for patient specific myeloid/blast markers; stained markers are shown in white. (G-I) IHC for CXCR4 in the corresponding BM samples. (A, D, G) Patient #1. (B, E, H) Patient #4. (C, F, I) Patient #10.

ular differentiating osteoblasts, has recently been shown to counteract the induction of apoptosis within the leukemic compartment which can be triggered by CXCL12 ligation to CXCR4.[42,43] Against this background, it is currently unclear what impact CXCR4 targeting by small molecule CXCR4 antagonists or monoclonal antibodies will have in the clinic, and, in particular, on eliminating the LICs that fundamentally contribute to relapse. Despite this mechanistic uncertainty, the first-in-class CXCR4 inhibitor AMD3100 (Plerixafor) has been tested as a chemosensitizing agent in relapsed or refractory AML in a phase I/II trial with encouraging preliminary results.[44] Further trials involving monoclonal antibodies and novel CXCR4-targeting small molecule inhibitors such as BL-8040 are under way (*EudraCT 2014-002702-21*). Disrupting ligand-mediated CXCR4 downstream activity by antagonists is one approach currently being tested. Physically targeting the BM niche characterized by the CXCR4-CXCL12 interaction could be an attractive alternative. One highly interesting method that provides such physical targeting is peptide receptor radionuclide therapy (PRRT). PRRT has been successfully integrated into the therapeutic algorithm of neuroendocrine tumors (NETs).[45] It usually involves the diagnostic imaging of the receptor to ensure target expression, followed by the application of a therapeutically labeled peptide (e.g. Lutetium-177 octreotate), thus constituting a theranostic procedure. In patients with AML, an endoradiotherapeutic approach with CD45 as target has been successfully tested in a phase I/II trial in the conditioning regimen prior to allogeneic SCT.[46] For such a purpose, the data presented within our CXCR4 examinations represent an important step, as they show that, at least in a subgroup of patients, there is a substantial expression of CXCR4, and that AML can even be imaged using the novel CXCR4-specific molecular PET probe Pentixafor. Pentixafor has already been labeled with therapeutic radionuclides such as ^{90}Yttrium and 177Lutetium, and compassionate use therapies have been applied to patients with very advanced multiple myeloma.[25] A phase I/II study in myeloma using the CXCR4-directed theranostic approach is currently under investigation (*EudraCT 2015-001817-28*). With regard to AML, however, it is still not at all clear whether measurable high CXCR4 expression is a prerequisite for such a therapy, since it can be assumed that targeting the niche *via* CXCR4 could have an effect on all hematopoietic cells harbored there. The imaging data presented in our study reveals crucial information on *in vivo* CXCR4 expression in

myeloid malignancy. Although we still have no data on ALL, very recent work defines the CXCR4/CXCL12 interaction as crucial for disease maintenance and progression in ALL.[18,19]

We are continuing to learn more about both the molecular and the genetic characterization of AML and ALL.[47] Thus, markers for detecting MRD are available that provide high sensitivity,[48] avoiding the need for additional imaging. We foresee the major application of CXCR4 targeting using the herein described CXCR4-binding peptide within a theranostic approach, i.e. as a conditioning regimen within an allogeneic SCT. The importance of the CXCR4/CXCL12 axis as a label of the LIC niche, as well as the observation that relapsed leukemias frequently express high levels of CXCR4, makes radiolabeled CXCR4 targeting an attractive novel therapeutic approach.

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Summary

Targeted positron emission tomography imaging of CXCR4 expression in patients with acute myeloid leukemia

Because CXCR4 has been described to be expressed and even of prognostic significance in AML, our primary aims in this project were to systematically assess CXCR4 surface expression in AML and MDS patients treated at our institution and test if CXCR4-directed imaging with ^{68}Ga-Pentixafor is feasible in AML. Initially, a flow cytometry protocol for CXCR4-surface expression was designed and optimized to measure CXCR4 expression on leukemic blasts in bone marrow samples of AML and MDS patients. We found that in a subset of patients, CXCR4 is highly expressed on malignant AML cells and that patients with blast counts greater than 30% of all bone marrow cells had significantly increased CXCR4 expression. Furthermore, we measured CXCR4 surface-expression and transcript levels on 11 different AML cell lines and found that mRNA levels correlate with surface-expression. Based on these data, we selected 3 cell lines CXCR4 surface levels ranging from very low/absent (KG1a) to high (OCI-AML3) for in-vivo testing of the CXCR4-directed imaging tracer ^{68}Ga-Pentixafor. To this end, we generated subcutaneous xenografts in immunocompromised NOD/SCID mice and performed Ga-Pentixafor PET imaging as soon as tumors were palpable. The CXCR4 expressing cell lines OCI-AML3 and NOMO-1 showed uptake of the tracer, whereas KG1a did not. To prove that uptake of Ga-Pentixafor is exclusively CXCR4-dependent we knocked out CXCR4 using a CRISPR-Cas9 approach in OCI-AML3 cells and could show that CXCR4-deficient xenografts were lacking Ga-Pentixafor accumulation. Finally, we performed CXCR4-directed PET/MRI in 10 AML patients and could show that both medullary and extramedullary leukemic burden could be visualized by Ga-Pentixafor and that patients without hematological neoplasms did not have excessive background signal in the bone marrow.

Taken together, we could show that CXCR4-directed PET/MRI is feasible both preclinically in AML xenograft models and clinically in patients suffering from AML.

I designed and performed all cell-line and mouse experiments, developed the protocol for measuring CXCR4 on the cell surface, analyzed and visualized all flow cytometry data shown and wrote the manuscript.

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Research Paper

Dual Targeting of Acute Leukemia and Supporting Niche by CXCR4-Directed Theranostics

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Abstract

C-X-C chemokine receptor 4 (CXCR4) is a transmembrane receptor with pivotal roles in cell homing and hematopoiesis. CXCR4 is also involved in survival, proliferation and dissemination of cancer, including acute lymphoblastic and myeloid leukemia (ALL, AML). Relapsed/refractory ALL and AML are frequently resistant to conventional therapy and novel highly active strategies are urgently needed to overcome resistance.

Methods: We used patient-derived (PDX) and cell line-based xenograft mouse models of ALL and AML to evaluate the efficacy and toxicity of a CXCR4-targeted endoradiotherapy (ERT) theranostic approach.

Results: The positron emission tomography (PET) tracer ^{68}Ga-Pentixafor enabled visualization of CXCR4 positive leukemic burden. In xenografts, CXCR4-directed ERT with ^{177}Lu-Pentixather distributed to leukemia harboring organs and resulted in efficient reduction of leukemia. Despite a substantial *in vivo* cross-fire effect to the leukemia microenvironment, mesenchymal stem cells (MSCs) subjected to ERT were viable and capable of supporting the growth and differentiation of non-targeted normal hematopoietic cells *ex vivo*. Finally, three patients with refractory AML after first allogeneic hematopoietic stem cell transplantation (alloSCT) underwent CXCR4-directed ERT resulting in leukemia clearance, second alloSCT, and successful hematopoietic engraftment.

Conclusion: Targeting CXCR4 with ERT is feasible and provides a highly efficient means to reduce refractory acute leukemia for subsequent cellular therapies. Prospective clinical trials testing the incorporation of CXCR4 targeting into conditioning regimens for alloSCT are highly warranted.

Key words: acute leukemia, microenvironment, C-X-C chemokine receptor 4, in vivo molecular imaging, theranostics

Introduction

C-X-C-motif chemokine receptor 4 (CXCR4) is a G-protein coupled transmembrane receptor that regulates a wide spectrum of physiologic processes in fetal organ development, hematopoiesis, and immune

system function. Knock-out studies in mice have demonstrated that absence of CXCR4 or CXCL12 – its only known chemokine ligand - is embryonically lethal [1, 2]. Binding of CXCL12 to CXCR4 initiates G-protein-dependent and -independent signaling events, including MAPK, AKT and ERK pathway activation, and Ca^{2+} release from the endoplasmic reticulum, which ultimately coordinates chemotaxis, homing, proliferation and cell survival [3]. CXCR4 is broadly expressed in the hematopoietic system; especially hematopoietic stem and progenitor cells (HSPCs) need CXCR4 for correct localization and retention in the bone marrow (BM) microenvironment. Therefore, the CXCR4/CXCL12 axis is indispensable for homeostasis of the HSPC pool in the BM [4]. CXCR4 is also commonly expressed or overexpressed in cancer cells, regulating proliferation, neo-angiogenesis, resistance to chemotherapy and metastasis to organs with high amounts of secreted CXCL12 [5, 6]. It has been shown for several cancer types, including acute myeloid leukemia (AML) that CXCR4 expression is associated with adverse prognosis [7]. Therefore, targeting CXCR4 with small molecule inhibitors or antibodies is being investigated in several clinical trials in various cancer types [8]. This concept is particularly promising in hematological malignancies, and preclinical studies have shown that CXCR4 inhibition can both kill cancer cells directly or dislocate them from their protective microenvironment, making them more susceptible to conventional chemotherapy in combined approaches [9-11].

In AML, malignant cells arising from immature myeloid progenitors or stem cells increasingly occupy the BM space as the disease progresses, leading to rapidly fatal complications without treatment. Even in patients receiving adequate intensive therapy, most commonly a combination of cytarabine and anthracyclines, overall survival rates 3 years after therapy range from 12-66% in younger and 3-33% in older adults, depending on prognostic factors [12]. Primary refractory disease and relapse after having achieved a complete remission are the biggest challenges in treating AML. Allogeneic stem cell transplantation (alloSCT) is considered the only curative option for these patients, and highly active conditioning regimens are needed to overcome resistance and reduce leukemic burden before transplantation. Due to the lack of a standard salvage induction regimen, choice of the preferred therapeutic strategy remains an individualized decision, often varying among different centers [13].

Acute lymphoblastic leukemia (ALL) is the most common cancer in childhood, with cure rates over 95% in low risk early B-cell lineage ALL patients [14]. However, ALL in adults is more difficult to treat and outcomes are worse than in pediatric patients [15]. With the bispecific (CD19, CD3) antibody blinatumomab and chimeric antigen receptor (CAR) T-cells emerging as effective treatments in relapsed B-ALL [16], comparably effective novel treatment strategies in T-ALL have been lacking so far [17, 18].

In both AML and ALL, the BM microenvironment is believed to play an essential role in protecting leukemic cells from chemotherapy or targeted therapies. This protective activity is believed to be a major determining factor in the survival of malignant cells and relapsing disease [11, 19, 20]. The niche is a major source of CXCL12 and this chemokine has been shown to induce stem cell quiescence, which contributes to resistance of leukemic stem cells to chemotherapy [21].

We have previously shown that CXCR4-directed PET imaging with the novel, human-specific CXCR4-binding peptide tracer ^{68}Ga-Pentixafor enables the visualization of CXCR4-expressing cells in AML and multiple myeloma patients [22, 23]. A modified version of Pentixafor, named Pentixather, allows labeling with β-emitting radionuclides (^{177}Lutetium, ^{177}Lu; ^{90}Yttrium, ^{90}Y) routinely used in clinical practice for cancer radiotherapy. This facilitates the possibility of a theranostic approach by combining CXCR4-directed imaging to select patients for CXCR4-directed endoradiotherapy (ERT) with Pentixather. This strategy would also allow targeting of the malignant cell-supportive BM microenvironment supporting malignant cells by cross-fire irradiation, which is particularly relevant in AML and ALL [11, 19, 20].

Here, we further develop this concept and apply a theranostic approach using ERT with Pentixather in preclinical models of T-ALL and AML, and ultimately in patients with relapsed AML after first alloSCT, who did not respond to conventional therapies. We investigate efficacy and toxicity, especially to the BM microenvironment, of this novel approach to treating leukemia, which provides crucial information for future prospective clinical studies.

Results

CXCL12/CXCR4 signaling is crucial for ALL and AML establishment

The CXCR4/CXCL12 axis is known to be involved in disease initiation, migration and treatment resistance in murine AML and ALL models, and in patients [20, 24]. We generated T-ALL (ALL0 and ALL230) and AML (AML356 and AML346) PDX mice (Fig. S1, S2 and Table S1) and used an orthotopic cell line xenograft model of human AML with

moderate or enforced CXCR4 expression (OCI-AML3-eGFP and OCI-AML3-CXCR4, Fig. S5a, b). To evaluate if the CXCR4/CXCL12 axis is functionally relevant in ALL and AML xenografts, we performed transwell migration assays with AML and ALL cells. CXCL12-induced migration and inhibition of CXCR4 with the clinically approved CXCR4 inhibitor AMD3100 (Plerixafor) resulted in significant reduction of the migrated cell fraction in OCI-AML3 cells, and in ALL0 and ALL230 PDX leukemia (Fig. 1a). Furthermore, immunoblot analysis confirmed that phosphorylation of AKT, a known downstream target and surrogate marker for CXCR4 activation, was induced (Fig. S3). To test if *in vivo* homing of human leukemic blasts to the BM and spleen of NSG mice is CXCR4-dependent, we pre-incubated T-ALL PDX (ALL230 and ALL0) and OCI-AML3 with the established CXCR4-blocking antibody 12G5 or an appropriate isotype control antibody, and injected these cells into NSG mice (Fig. 1b). After 48 h, BM and splenic infiltration by human leukemic cells was significantly lower when CXCR4 was blocked with 12G5, indicating that CXCR4-mediated homing is necessary for disease initiation in these acute leukemia models (Fig. 1c).

Thus, these leukemia models are suitable for testing CXCR4-directed theranostics *in vivo* with regard to efficacy and microenvironment effects.

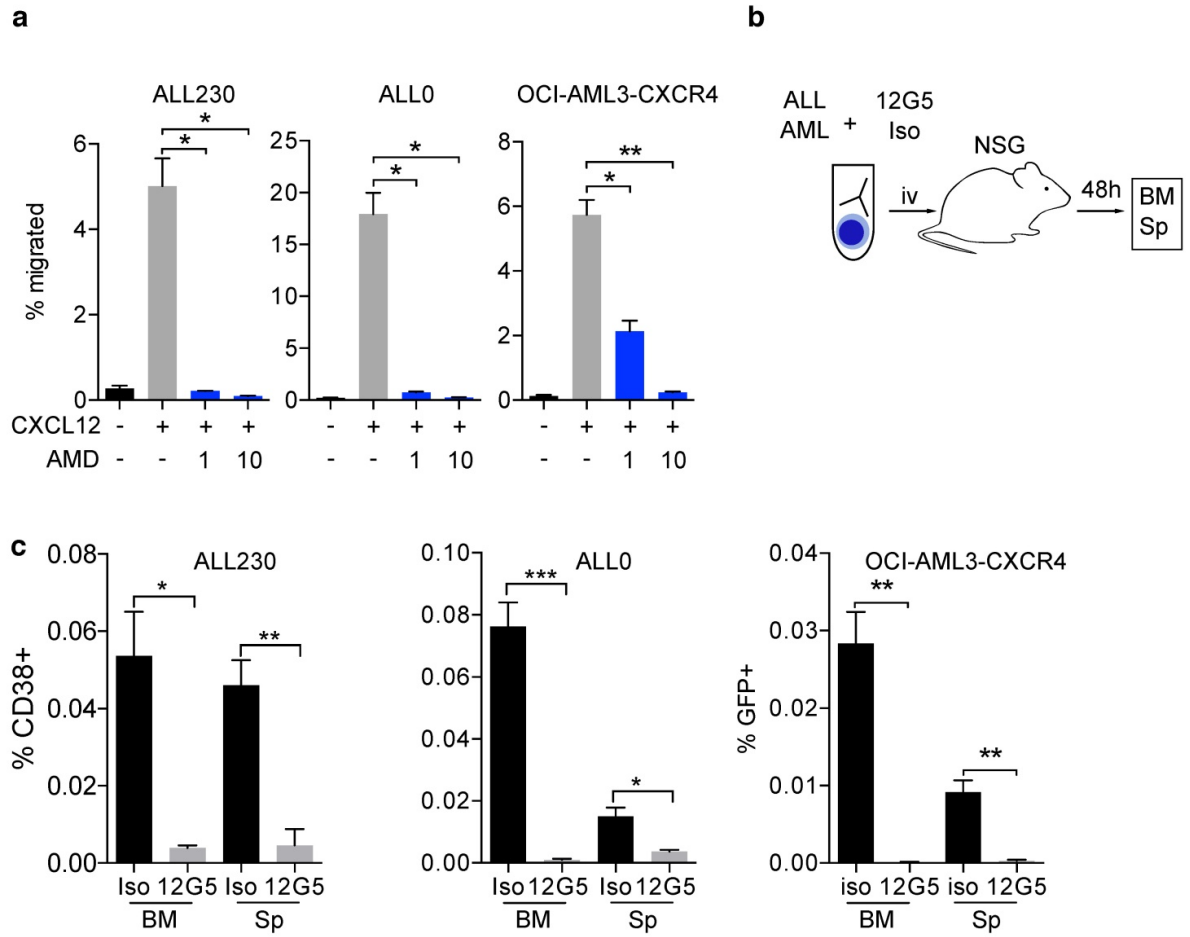

Figure 1. CXCR4/CXCL12 signaling is crucial for establishing acute leukemia PDX. a) Migration of OCI-AML3-CXCR4 and ALL PDX (ALL230 and ALL0) towards 100ng/µl CXCL12 alone or combined with 1µM and 10µM AMD3100 (n=3 replicates). **b)** Schematic of experimental setup for experiments shown in c). **c)** OCI-AML3-CXCR4 and ALL PDX homing in vivo after pre-incubation with CXCR4 antibody clone 12G5 or isotype control. Cells were injected into NSG mice and infiltration of organs was assessed after 48h (n=3 mice per group). Statistical significance was determined by two-sided t-tests, *p<0.05, **p<0.01, ***p<0.001, AMD: AMD3100, BM: bone marrow, Sp: spleen, NSG: NOD-SCID-gamma.

In vivo molecular CXCR4 imaging reflects surface expression of CXCR4 and represents the first step in CXCR4 theranostics

We next sought to determine if leukemic PDX cells expressing CXCR4 could be imaged *in vivo* with the human-specific CXCR4-binding peptide PET tracer ^{68}Ga-Pentixafor as an initial component of CXCR4 theranostics. The grafts of the employed PDX models of T-ALL and AML clearly displayed different levels of CXCR4 surface expression (Fig. 2a). Upon NSG recipient injection with PDX, peripheral blood (PB) blast counts were monitored with flow cytometry. ^{68}Ga-Pentixafor PET imaging was performed when human blasts could be detected in PB or when mice displayed symptoms of leukemia (weight loss, behavioral abnormalities). ^{68}Ga-Pentixafor enabled approximate visualization of leukemic burden of T-ALL (ALL230, ALL0) and AML PDX (ALL356) in spleens and bones of NSG mice (Fig. 2b) and correlated with CXCR4 surface expression of PDX (Fig. 2c). Histology and immunohistochemistry of imaged mice confirmed CXCR4 expression on human infiltrating blasts (Fig. 2d). ^{69}Ga-Pentixafor PET images of control NSG mice without leukemic burden are shown in Fig. S4 in different intensities.

To determine if Pentixather, the structurally modified therapeutic counterpart of Pentixafor, binds to human leukemia cells *in vivo*, we injected AML356 PDX recipients with ^{125}Iodine-Pentixather three, four and five weeks after injection of PDX cells. Binding of Pentixather to splenic AML blasts was detected by *ex vivo* autoradiographic imaging of spleens. Progressive splenic infiltration could be visualized by autoradiography, indicating that ^{125}I-Pentixather binds to AML356 PDX *in vivo* (Fig. 2e).

Thus, the CXCR4-directed therapeutic peptide Pentixather represents a pre-clinically applicable means to target human CXCR4+ PDX *in vivo*, and may serve as a surrogate imaging diagnostic for assessing human leukemia infiltration in BM and other organs.

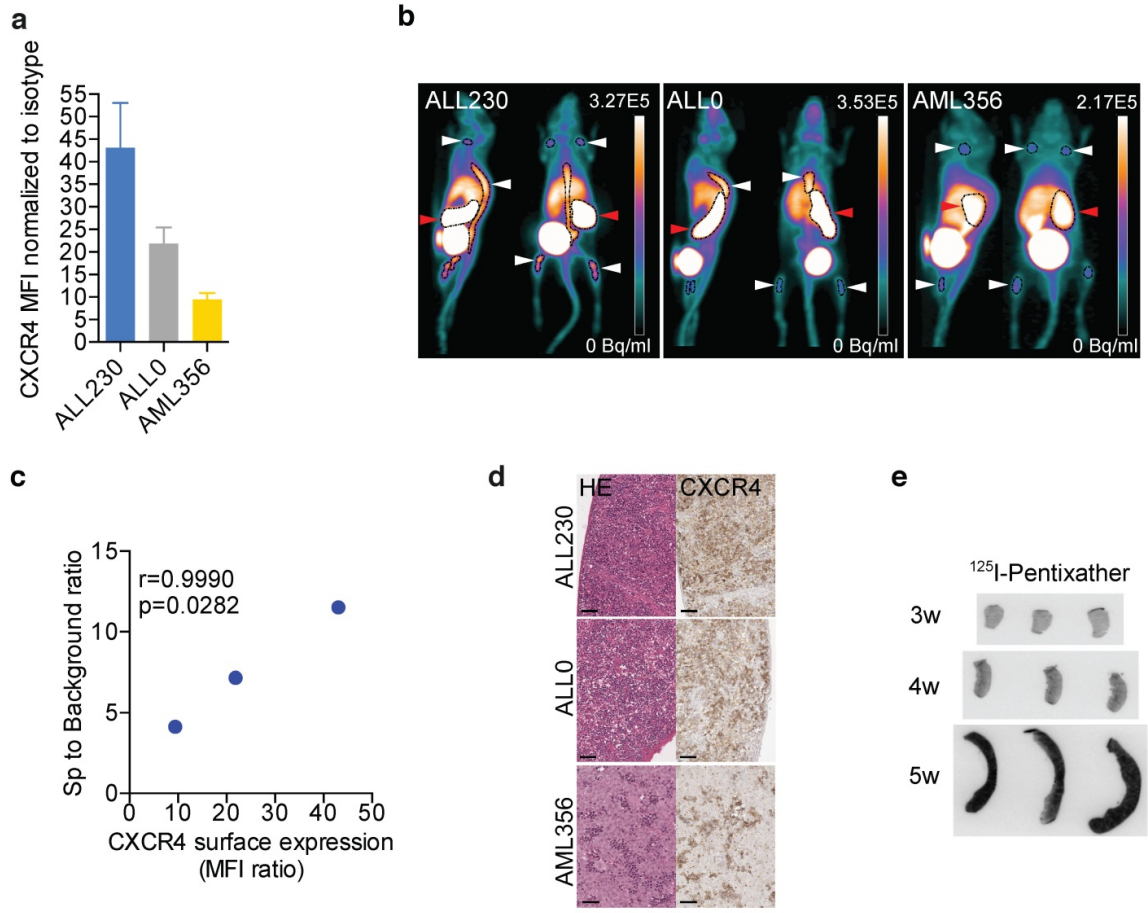

Figure 2. CXCR4 diagnostics in preclinical models of acute leukemia. a) CXCR4 surface expression in ALL and AML PDX (n=3 replicates). **b)** Representative PET images of ^{68}Ga-Pentixafor scans in mice bearing ALL and AML PDX. Red arrows: spleen, white arrows: bone marrow (n=6 ALL230, n=6 ALL0, n=5 AML356). **c)** Correlation between Ga-Pentixafor PET uptake and mean CXCR4 surface expression. **d)** HE staining and CXCR4 immunohistochemistry of spleens of ALL230, ALL0 and AML356 mice. Scale bars: 50 µm. **e)** Representative images of ^{125}I-Pentixather autoradiography of AML356 spleens. MFI: mean fluorescence intensity, HE: Hematoxylin and eosin, r: Pearson correlation coefficient.

Therapeutic CXCR4-targeted peptide effectively reduces leukemia in PDX mice

To test the efficacy of CXCR4 targeting *in vivo* we chose peptide labeling with the well-established therapeutic beta-emitter ^{177}Lu [25, 26]. Two CXCR4^{high} T-ALL PDX models and two cell line models of AML (OCI-AML-eGFP and OCI-AML-CXCR4) with different infiltration characteristics of BM, spleen and blood (Fig. S1) were subjected to ^{177}Lu-Pentixather (Lu-P) treatment (d0) or unlabeled Pentixather as control. Mice were sacrificed 3 and 7 days after injection of Lu-P (Fig. 3a). To determine the distribution of Lu-P in treated mice, we measured radioactivity in PB, BM and spleen of treated mice and found that Lu-P distributed to and strongly accumulated in leukemia-harboring organs (Fig. 3b).

In the ALL230 cohort, mice in the control group had large spleens (254.7 mg and 551.7 mg in the d3 and d7 groups, respectively). Lu-P therapy significantly reduced spleen size and weight in treated animals. In mice receiving the less aggressive ALL0 PDX, spleens were less enlarged ~4 weeks after PDX injection. Again, Lu-P therapy resulted in significantly reduced spleen weight as compared to control mice (Fig. 3c, d). In order to investigate ALL involvement of PB as well as spleen and BM, we performed flow cytometry. ALL230 displayed pronounced reduction of blast populations in spleen, BM and PB (Fig. 3e, f). Histology and immunohistochemistry of spleen and BM of control mice revealed an extensive infiltration with CXCR4^{+} neoplastic cells. In contrast, in all organs of Lu-P-treated mice, cellularity was significantly reduced and leukemic cells were effectively targeted by ERT (Fig. 3g). In BM and spleen of heavily infiltrated mice, although single neoplastic blasts were still detectable, hemorrhage and necrotic tissue damage were apparent, indicating effective targeting of the infiltrative tumor cell population as a result of therapy.

In summary, these experiments show that CXCR4-directed ERT effectively targeted CXCR4+ tumor cells and reduced leukemic burden in T-ALL PDX recipient mice. The data also indicate that damage occurred to the remaining functional BM, most likely as a result of cross fire effects by Lu-P.

To answer the question whether the intensity of CXCR4 surface expression affected treatment efficacy, we used OCI-AML3-CXCR4, with a ~2.5-fold overexpression of surface CXCR4 (Fig. S5a) as compared to empty vector control-eGFP transduced cells. These cells were injected intravenously into NSG mice to establish xenografts, followed by treatment with Lu-P or unlabeled Pentixather. Three days after treatment we observed a significant reduction of spleen weight irrespective of the level of CXCR4 expression (moderate vs. enforced) (Fig. S5c). Importantly, treatment of AML346, the PDX line with the lowest CXCR4 expression, did not result in reduction of leukemic burden (Fig. S6.). These experiments with AML models with low (AML346), moderate (OCI-AML3-eGFP) and elevated (OCI-AML3-CXCR4-eGFP) CXCR4 surface expression suggested that a certain degree of target expression, i.e., CXCR4 surface expression, is necessary for treatment efficacy. This finding should be relevant with regard to clinical benefit for AML patients with various extent of CXCR4 expression.

Cross-fire originating from Lu-P targeting impairs normal HSPCs and the BM niche

We hypothesized that, owing to the β-emitting properties of ^{177}Lu, radionuclide targeting of CXCR4 expressing leukemic cells would result in damage to the surrounding tissue, including murine HSPCs and other cellular components of the BM niche. The PDX models in combination with a targeting peptide specific for human CXCR4 [27, 28] thus provided an ideal model to address cross-fire effects on murine recipient HSPCs and the host cellular microenvironment.

To directly assess damage inflicted to murine HSPCs, we performed colony forming unit (CFU) assays with murine growth factors on BM harvests of ERT-treated and control ALL230 and ALL0 PDX mice (Fig. 4a). In both PDX models, the proliferative potential of murine BM HPCs was significantly reduced with treatment. In ALL230 mice, which had subtotal infiltration of the BM by human ALL (Fig. 3g), Lu-P treatment even resulted in complete ablation of CFU growth (Fig. 4b). Using flow cytometry, we observed a significantly reduced fraction of lineage negative (Lin-), Sca1 positive, cKit positive stem cells (LSKs), and BM myeloid progenitors (MPs) (Fig. 4c, gating strategy in Fig. S7). In Lu-P treated mice, LSKs and MPs were almost completely absent in flow cytometry, further emphasizing the toxicity of radioactive targeting for the neighboring hematopoietic population.

To test if the BM niche, especially the MSC population crucial for reconstitution of hematopoiesis, is affected by ERT, we isolated and analyzed endosteal niche cells from collagenase treated bones of Lu-P treated and untreated mice and performed flow cytometry analyses as described earlier [29, 30]. MSC were still present in both ERT-treated and control mice after isolation of endosteal niche cells (Fig. 4d). To further assess if the BM MSC population was still viable after Lu-P

treatment, we performed CFU-F assays to isolate MSCs from bones of treated and control mice (representative images in Fig. S8). MSCs are defined as adherent cells emerging from bones, which form colonies and proliferate *in vitro* [31]. Colony numbers were not significantly different in Lu-P vs. control with a trend towards reduction in the treatment group (Fig. 4e). Further growth of the isolated MSCs was significantly reduced in the Lu-P group, indicating targeting of the BM niche by the cross-fire effect (Fig. 4f).

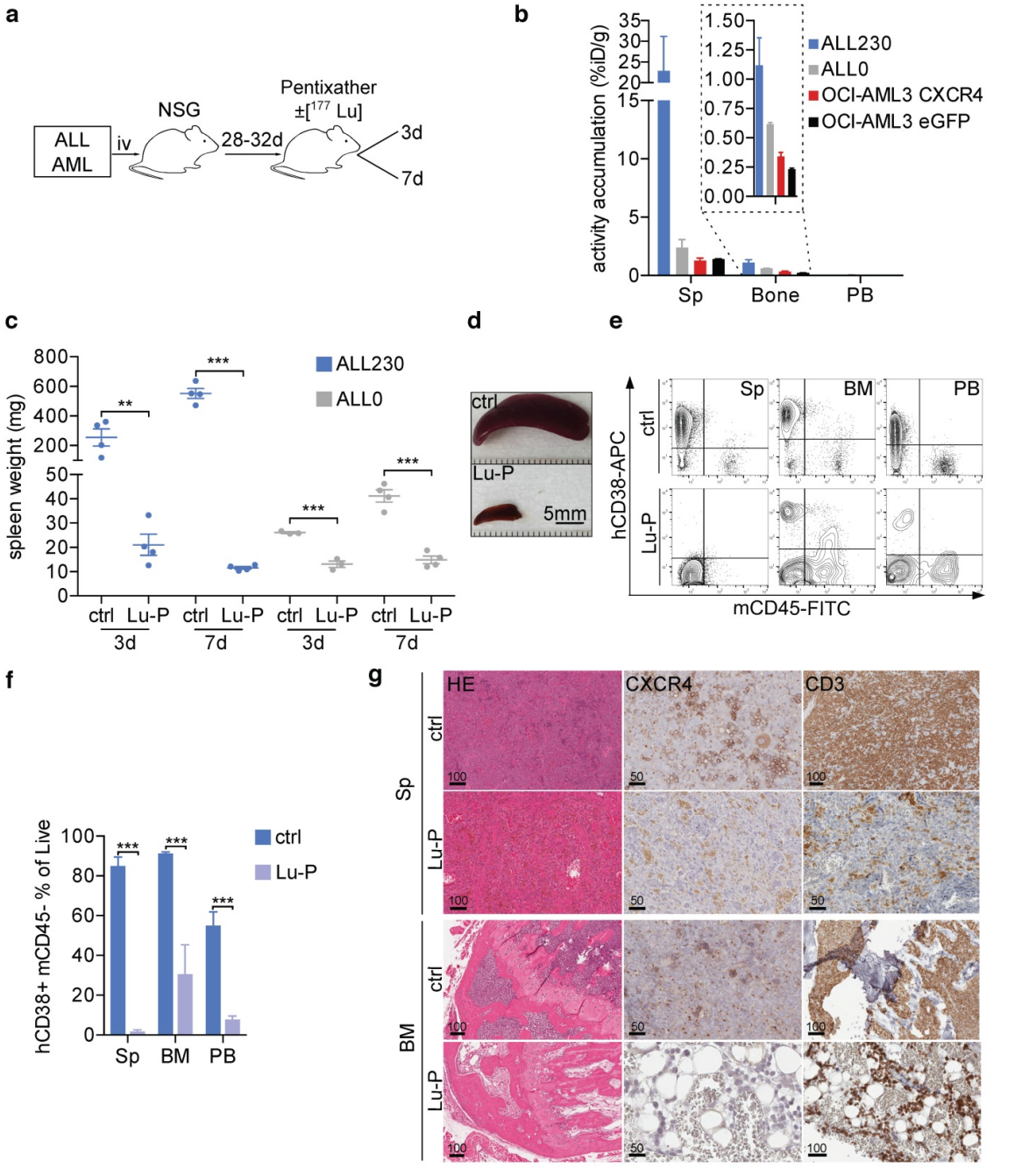

Figure 3. CXCR4-directed theranostics effectively reduce leukemia burden. a) Schematic of experimental setup. **b)** Activity accumulation in percent of injected dose per gram (%iD/g) of Sp, bone and PB 3d post injection of Lu-P in mice bearing ALL PDX or OCI-AML3 cells overexpressing CXCR4 or eGFP empty vector (n=4 for ALL230, OCI-AML3 CXCR4, n=3 for ALL0, OCI-AML3 eGFP). **c)** Spleen weight of ctrl and Lu-P treated ALL0 and ALL230 mice after 3 and 7d (n=3 for ALL0 3d, n=4 for all other groups). **d)** Representative images of ALL230 spleens treated for 3d with ctrl or Lu-P. **e)** Representative FACS plots of Sp, BM and PB of ALL230 mice treated for 7d with ctrl or Lu-P, gated on propidium-iodide negative, live cells. Human ALL cells are human CD38 positive and murine CD45 negative. **f)** Quantification of e), percentage of human blasts is shown. **g)** Sp and BM histology of ALL230 mice treated with ctrl or Lu-P with HE stain, CXCR4 and CD3 immunohistochemistry, scale bar length in µm as indicated. Statistical significance was determined by two-sided t-tests, *p<0.05, **p<0.01, ***p<0.001, ctrl: control, Lu-P: 177Lu-Pentixather, PB: peripheral blood.

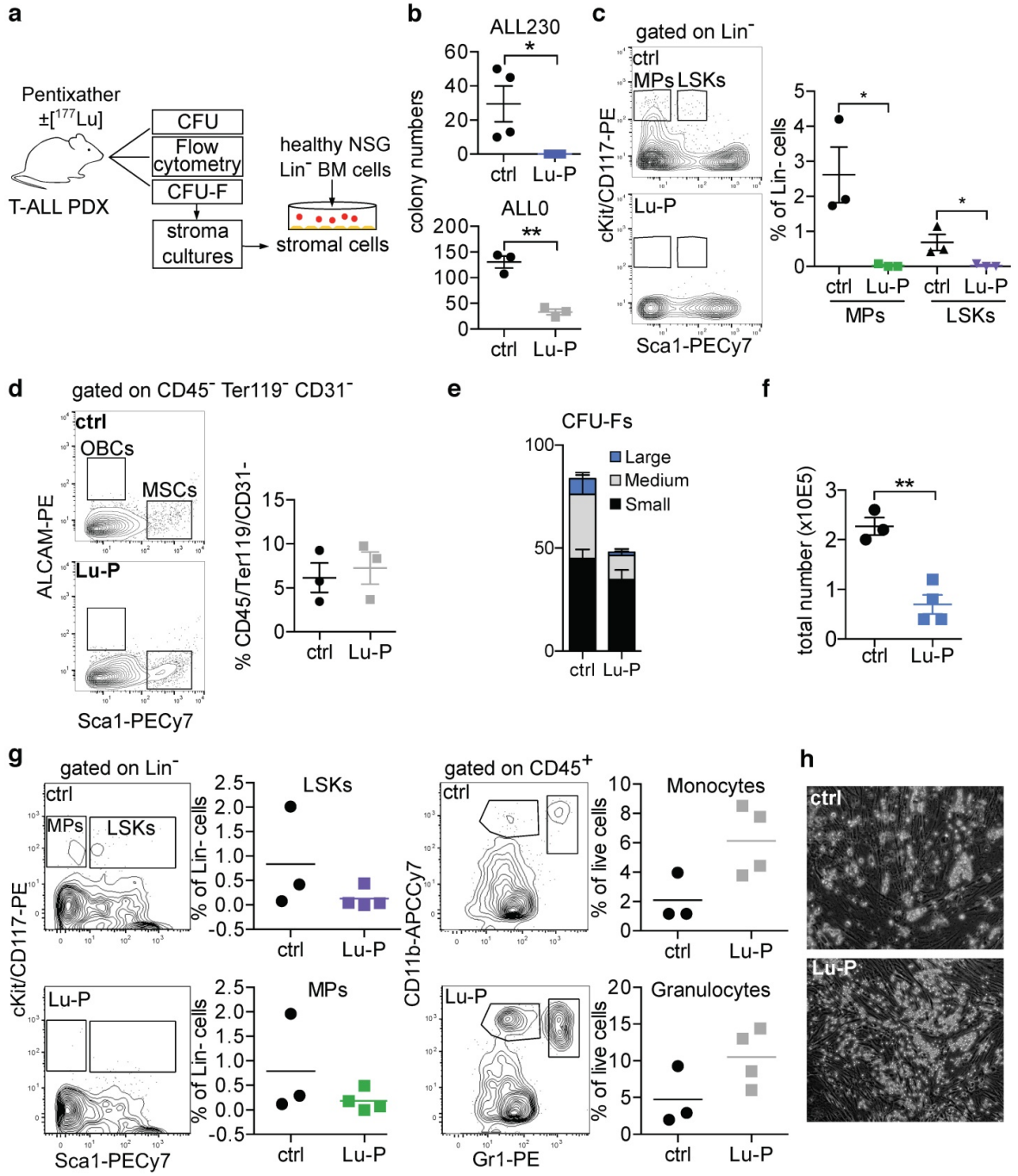

Figure 4. Cross-fire effect on non-malignant hematopoiesis and BM microenvironment. a) Schematic of experimental setup. **b)** Methylcellulose CFU assay for murine progenitors of BM from ALL230 and ALL0 mice 3d after treatment with ctrl or Lu-P (n=4 mice for ALL230, n=3 mice for ALL0). **c)** Flow cytometry of BM isolated from ctrl and Lu-P treated ALL0 mice. LSKs and MPs were analyzed (n=3 mice per group). **d)** Comparison of MSCs (Sca1+, ALCAM- fraction of CD45-, Ter119-, CD3-, live cells) of treated and control ALL0 mice (n=3 mice per group). **e)** Quantification of CFU-F colony numbers (small: <20 cells, medium: 20-200 cells, large >200 cells) of ctrl and Lu-P treated ALL230 mice (n=4 mice per group). **f)** Total number of stromal cells from d) after 18d of culture. **g)** Coculture of MSCs from Lu-P and ctrl ALL20 mice (3d) and lineage- BM cells from WT NSG mice. LSKs, MPs, Gr1+CD11b+ granulocytes and Gr1^{med}CD11b+ monocytes were analyzed after 7d of coculture (n=3 for ctrl, n=4 for Lu-P). **h)** Representative microscopic images of coculture experiment as described in g). Statistical significance was determined by two-sided t-tests, *p<0.05, **p<0.01, ***p<0.001, colony forming unit (CFU), LSKs: Lineage-, Sca1+, cKit+ cells, MPs: myeloid progenitors, MSCs: mesenchymal stem cells, CFU-F: colony forming unit-fibroblasts.

Support by the BM niche is essential for engraftment of HSPCs after myeloablative conditioning. We therefore investigated whether the isolated MSCs were still supportive of healthy HSPCs by co-culturing MSCs with MACS-purified immature (Lin-) murine HSPCs and analyzed MP and LSK

number and frequency, and differentiation into mature myeloid cells. In both Lu-P treated and control stroma co-cultures, MPs and LSKs were supported and differentiation into granulocytes and monocytes was induced (Fig. 4g). Furthermore, HSPCs in co-culture formed cobblestone-like areas on both Lu-P-treated and control stroma (Fig. 4h). To model the effect of Lu-P on human BM MSCs, a small subset of which are known to express CXCR4 [32], we isolated MSCs from BM samples of healthy individuals and treated MSCs with Lu-P or unlabeled Pentixather. After treatment, we co-cultured purified healthy human CD34+ cells with the MSCs and assessed viability and potential to form colonies in methylcellulose (Fig. S9). There was no significant impairment of Lu-P pre-treated MSCs' ability for subsequent support of normal CD34+ cells.

In summary, the cross-fire effect caused by β-emitter ERT results in significant damage to HSPCs and substantially targets proliferative activity of BM niche cells, while the functional capacity of stromal cells to support the growth and differentiation of in vitro co-cultured healthy immature hematopoietic cells was not abrogated.

Pentixather treatment in very advanced human acute leukemia: ERT followed by second alloSCT

Finally, the concept of targeting CXCR4 in acute leukemia by ERT was translated into a clinical setting. Three patients with relapsed AML were referred for assessment of further treatment option, e.g., Pentixather treatment. All patients were heavily pretreated, including first alloSCT, but had experienced early relapse (for patient characteristics and previous treatments see Table S2). Given the lack of alternative treatment options in this advanced disease stage, experimental CXCR4-directed treatment was offered on a compassionate use basis (German Drug Act, §13,2b) in compliance with §37 of the Declaration of Helsinki. All subjects gave written informed consent prior to therapy.

Patient #1 (46-year-old male) presented with AML without maturation (AML M1). After standard induction chemotherapy and salvage therapy due to primary refractory disease first alloSCT was performed. Three months after SCT, relapse was diagnosed and the patient was referred. Given the early relapse, investigation of CXCR4 expression as a putative therapy target was considered. ^{68}Ga-Pentixafor-PET demonstrated receptor expression (Fig. S10a), which qualified for CXCR4-targeted ERT. Pre-therapeutic dosimetry resulted in tolerable activities of 5 GBq (with the kidneys being the dose-limiting organ) of ^{90}Y-Pentixather (Y-P), and achievable BM doses of ~11 Gy. ^{90}Y as radionuclide was chosen due to its higher energy and longer range as compared to ^{177}Lu, and because its shorter half-life allows earlier SCT. ERT with 4.72 GBq of Y-P was performed and well tolerated. In order to maximize the therapeutic effect of ERT, we decided to increase internal radiation by adding a course of ^{153}Sm-labelled ethylene diamine tetramethylene phosphonate (EDTMP), which, in contrast to Pentixather, localizes to the surface of cortical and trabecular bone, to the conditioning regimen. Based on previous studies investigating ^{153}Sm-EDTMP for BM ablation [33, 34], 15.9 GBq was intravenously administered five days after Y-P and resulted in additional BM irradiation as high as 8 Gy for the red marrow and 53 Gy in osteogenic cells in the cortical bones. Conditioning was completed by fludarabine (40 mg/m^2; d-8 to d-5), thiotepa (5 mg/kg body weight; d-4; twice), melphalan (70 mg/m^2; d-3 to d-2) and antithymocyte globulin (ATG; 10/20/30 mg/kg; d-10 to d-8). 19 days after the initial Y-P treatment, the patient received a second alloSCT (day 0). Besides prolonged epistaxis in aplasia requiring temporary intubation, he did not experience major complications. On day +11, recovery of neutrophils, on day +14, recovery of thrombocytes could be recorded (Fig. 5d). On day +30, donor chimerism in peripheral blood was 99.89%.

Patient #2 (67-year-old female) suffered from therapy-related AML (t-AML) that arose after exposure to polychemotherapy for breast cancer 18 years earlier. After induction therapy and first alloSCT (Table S2) the patient was in complete remission for 16 months until relapse. At the time of presentation, 50% AML infiltration of the BM as well as multiple extramedullary disease (EMD) lesions in the soft tissue of the pelvis and abdomen were present. ^{68}Ga-Pentixafor PET revealed a rather modest receptor expression of the BM but intense tracer uptake in all EMD lesions (Fig. S10b). Given the lack of established treatment options, a second alloSCT after a combined conditioning approach using Y-P for both the intra- as well as especially EMD lesions (administered activity of 4.5 GBq; d-20), ^{188}Re-labelled anti-CD66 antibodies for BM ablation (5.2 GBq; d-14) and conventional agents including rituximab (375 mg/m^2; d-6), total body irradiation (TBI, 2 Gy; d-5); ATG (10 mg/kg; d-4; resulting in anaphylactic shock), and melphalan (70 mg/m^2; d-3 to d-2) was chosen. Pre-therapeutic dosimetry in this patient yielded estimated BM doses of up to 17 Gy and EMD doses of 23 Gy. Achieved doses for ^{188}Re-anti-CD66 antibodies could not be calculated due to technical problems; however, post-therapeutic whole-body scintigraphy proved high antibody retention in the bone marrow

(Fig. S10b). Unfortunately, this patient died after hematological recovery from septic complications on day +17 after second SCT (Fig. 5d). Chimerism analysis had not yet been performed.

Patient #3 (39-year-old male) had been diagnosed with AML M0 nine months prior to presentation (Table S2). He had experienced leukemia relapse only five months after haploidentical first alloSCT (Fig. 5a-c) and was referred for salvage therapy. In analogy to patient #2, combined CXCR4-directed ERT (with 2.7 GBq of Y-P; d-33) and anti-CD66 therapy (with 7.7 GBq of ^{188}Re-anti-CD66 antibodies; d-28) as part of conditioning prior to second alloSCT was performed and resulted in red BM doses of 20 Gy. During aplasia, severe mucositis and a septic episode could be successfully managed. After neutrophil reconstitution on d+20, 99.9% peripheral blood donor chimerism were recorded on d+26. The patient was discharged the day after. Repeated BM biopsy 6 months after alloSCT confirmed complete remission.

Discussion

Our data provide first evidence for efficacy of CXCR4-directed ERT with Pentixather in preclinical models of T-ALL and AML, and a limited number of patients treated within individual therapy approaches for very advanced disease.

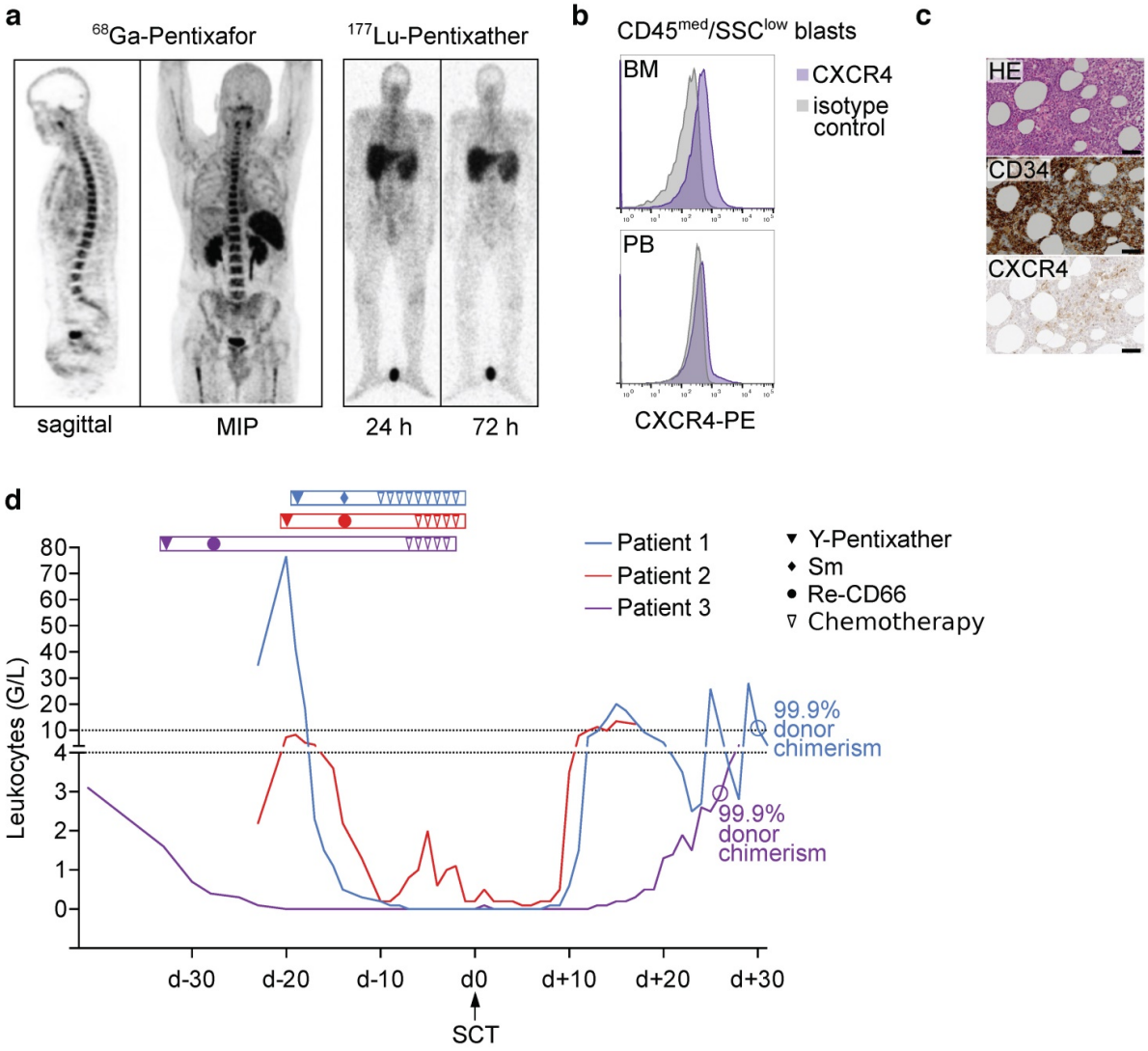

Figure 5. Proof-of-concept evaluation in advanced human acute leukemia: ERT followed by allogeneic stem cell transplantation. a) ^{68}Ga-Pentixafor PET imaging and planar whole-body scintigraphic images 24h and 72h after injection of 200 MBq Lu-P in patient 3 (activity injected for pre-therapeutic dosimetry). **b)** Blast CXCR4 surface expression in flow cytometry in patient 3 (gated on CD45^{med}/SSC^{low} blasts) **c)** HE stain, CD34 and CXCR4 BM immunohistochemistry of patient 3, scale bars: 50 µm. **d)** Leukocyte counts and % donor chimerism of patients undergoing Y-Pentixather based conditioning and allogeneic stem cell transplantation. MIP: maximum intensity projection, Sm: ^{153}Samarium-EDTMP, Re-CD66: ^{166}Rhenium-anti-CD66 antibody.

When AML or ALL relapse or are refractory to treatment, a multitude of challenges arises. Both relapse and refractoriness are a manifestation of cells having developed resistance to therapy or re-emerging after a period of dormancy. In such a scenario, salvage therapy and pre-transplant conditioning needs to be of the highest possible efficacy with regard to eradicating leukemic cells without compromising engraftment or causing excessive or irreversible damage to non-hematopoietic organs, which would further increase treatment-related morbidity and mortality.

Despite the prominent role of cell-intrinsic mechanisms in T cell as well as myeloid HSPC transformation, ALL/AML cell growth is not fully cell autonomous. In the BM, T-ALL lymphoblasts establish stable contacts with vascular endothelial niche cells expressing CXCL12 and are dependent on cues from the microenvironment for cell proliferation and survival [20, 35, 36]. A similar role for the CXCR4/CXCL12 axis has been established for AML [11, 19, 37], indicating that CXCR4/CXCL12 activity in the BM microenvironment marks a highly beneficial local sanctuary for ALL and AML cells. Despite tremendous efforts and the fact that several drugs are available and have already entered the clinical phase of development, neither small molecule or peptide inhibitors nor CXCR4-targeted antibodies have yet shown convincing efficacy [38]. There are several reasons why such pharmacological approaches (antibodies, inhibitors) may not result in long-term benefit. Potential mechanisms of resistance include downregulation or internalization of surface CXCR4, heterogeneous expression on cancer cells resulting in incomplete targeting, and potential competition with locally increased CXCL12 concentrations [39, 40].

Our therapeutic preclinical PDX data indicate that the CXCR4 ERT concept provides substantial efficacy via the cross-fire effect, overcoming the requirement that every single cancer cell is reached und thus also providing a strong niche-targeted impact. This however comes with a major intricacy, namely targeting basically all niches where CXCR4-expressing cells, whether malignant or not, reside. Due to the limited number of patients treated with the experimental protocol presented in this work, and the fact that only AML patients received Pentixather ERT, we cannot conclude at this time whether the level of expression of CXCR4 is predictive of efficacy or toxicity. The preclinical data regarding level of CXCR4 expression (Fig. 2a) and correlation of CXCR4 expression with ^{68}Ga-Pentixafor PET imaging however could hint towards a correlation between organ-bound dosage and local effectiveness as well as toxicity (Fig. 3, Fig. 4, Fig. S9).

Direct and indirect targeting of the interaction of leukemic cells with cellular components of the BM microenvironment represents an attractive strategy to induce the best possible remission before immunological intervention. In fact, various studies have shown that AML patients who enter conditioning and alloSCT have significantly better survival as compared to those with residual disease [41]. Severe impairment of the hematopoietic niche before alloSCT may result in severely prolonged pancytopenia or engraftment failure. Therefore, careful dosage and detailed preclinical evaluation of a potential niche-targeting agent is warranted. Our study of the BM microenvironment of PDX recipient mice, whose HSPC and MSC cannot bind Pentixather, and our experiments with human MSC showed that the number of niche cells is not affected by treatment. However, proliferation of niche cells after in vivo ERT is severely impaired. Despite this strong collateral damage observed within the BM microenvironment of PDX recipient mice, whose HSPC and MSC cannot bind Pentixather, patients receiving radionuclide-labeled Pentixather showed engraftment well within the expected range. Clearly, only a prospective clinical trial will allow determining the full extent of short and long-term effects of Pentixather treatment on the BM niche and other organs. Our results suggest that despite proliferative impairment, recipient MSC are well capable of supporting HSPC maintenance when cultured *ex vivo*.

Uptake of the imaging tracer Pentixafor within the liver of mice undergoing ^{68}Ga-Pentixafor PET imaging is not reflected in patients [42]. Due to the high affinity to human compared to murine CXCR4 [28], this phenomenon is most likely due to unspecific binding or an idiosyncrasy of the murine metabolism of Pentixafor.

TBI has been applied in pre-transplant regimens as early as the 1960s, and is still used routinely in the treatment of leukemia due to its efficacy and ability to penetrate sanctuary sites [43]. Toxicity to non-hematopoietic organs is a major limitation of this technique. Approaches delivering radioactivity directly and selectively to the hematopoietic system, for instance radiolabeled antibodies against CD45 or CD66, have been used in pre-transplant conditioning [44, 45]. Targeting CXCR4 with a radioactive peptide might facilitate killing particularly therapy-resistant and otherwise difficult to treat leukemic cells in their protective niche. Especially in T-ALL, CXCR4 was recently shown to be essential for leukemia-initiating cells and maintenance of the disease in the BM [20, 24], further supporting potential benefit of CXCR4 targeting in this malignancy. We would expect that

the same applies for CXCR4+ AML. We also would expect that Pentixather ERT could be provided to patients in need of non-chemotherapy salvage therapy before alloSCT who are not candidates for full-dose TBI due to age and/or comorbidities.

Due to the flexibility and various possibilities to label Pentixather, the choice of the radionuclide is not limited to ^{177}Lu or ^{90}Y [26]. Alpha emitters like ^{213}Bismuth or ^{225}Actinium have a much shorter range and deliver higher amounts of energy, potentially increasing specificity of targeting tumor cells and their immediate surroundings [46, 47]. We chose ^{177}Lu in animal experiments and ^{90}Y in patients due to different maximum ranges (0.8 mm and 11 mm, respectively), with the rationale to deliver appropriate doses to affected organs and spare healthy tissue. Other theranostic strategies currently being used in clinical practice include the treatment of midgut neuroendocrine tumors using ^{177}Lu-DOTATATE, with excellent results in a phase 3 trial [48].

For a CXCR4-directed theranostic concept, screening with ^{68}Ga-Pentixafor PET/CT or MRI will determine patients eligible for incorporation of Pentixather treatment in the conditioning regimen. A clinical phase I/II study (COLPRIT, EudraCT: *2015-001817-28*) to evaluate safety of a Pentixafor/Pentixather based concept in the treatment of relapsed/refractory Non-Hodgkin lymphoma and multiple myeloma followed by autologous SCT is planned to be performed. Whether CXCR4 PET imaging is required in leukemia or can be replaced by flow cytometry of BM needs to be evaluated in the controlled prospective clinical setting. Based on the data presented here however, a clinical trial testing the incorporation of Pentixather into conditioning regimens before alloSCT is urgently warranted.

Materials and Methods

Cell culture and cell lines

The human AML cell line OCI-AML3 was cultured in high glucose (4.5 g/L) DMEM supplemented with 10% FCS, 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Leibniz, Germany) and routinely re-authenticated. Cells were maintained at 37°C in a 5% CO_2 humidified atmosphere. All media and supplements were obtained from Gibco/Life Technologies (Carlsbad, CA, USA). CXCR4 overexpression was achieved by lentiviral transduction of OCI-AML3 cells with pHIV-CXCR4-eGFP, with cDNA of human CXCR4 cloned into pHIV-eGFP (Addgene plasmid ID #21373).

Migration assay

Migration assays were performed as described previously [23]. Briefly, cells were incubated with AMD3100 (Selleckchem, Houston, TX, USA) or DMSO and placed in the top chamber of transwell plates with 5 µm pore size (Corning Inc., Corning, NY, USA) with 100 ng/mL CXCL12 in the lower chamber (R&D Systems, Minneapolis, MN, USA). Cells were then incubated at 37°C for 4 h and the total number of cells migrated to the lower chamber was measured using CountBright beads (Thermo Fisher, Waltham, MA, USA).

CFU, CFU-F and MSC coculture

For CFUs, BM of treated and untreated mice was mixed with methylcellulose with murine growth factors M3434 (Stemcell Technologies, Vancouver, Canada) and processed according to the manufacturer's protocol.

CFU-Fs (MSCs) were isolated by placing bone fragments in stromal cell culture medium and cultured on plastic surfaces coated with 0.1% gelatin as described earlier [49]. Lineage negative cells (2500 total) from BM of NSG mice were isolated by MACS (magnetic cell separation, lineage depletion kit, Miltenyi Biotec, Bergisch Gladbach, Germany) and cultured on confluent MSCs for 7 days.

Flow cytometry

Experiments were performed on a Cyan ADP (Beckman Coulter, Brea, CA, USA). For surface markers used in routine diagnostics (Fig. S2), a Cytomics FC500 flow cytometer was used (Beckman Coulter). The following antibodies were used: human CD3-FITC (clone UCHT1), human CD4-PECy5 (clone 13B8.2), human CD5-PECy7 (clone BC1a), human CD7-PECy5 (clone 8H8.1), human CD8-ECD (clone SFC/Thy2D3), human CD13-PE (clone SJ1D1), human CD14-PE (clone RM052), human CD15-FITC (clone 80H5), human CD30-PE (clone HRS-4), human CD33-PE (clone D3HL60.251), human CD34-FITC (clone 581), human CD56-PE (clone N901(NHK-1)), human CD64-FITC (clone 22), human CD117-PE (clone 104D2D1), human HLADR-ECD (clone Immu357), human MPO-FITC (clone CLB-MPO-1), human TdT-FITC (HT1,HT4,HT4,HT9) from Beckman Coulter; human CXCR4-PE/BV421 (clone 12G5), BV421-isotype, human CD38-APC (clone HB7) from BD Biosciences (Franklin Lakes, NJ, USA), human CD45-eFluor450 (clone HI30), murine CD45-FITC/APC-eFluor780 (clone 30F11), murine CD3-PECy5.5 (clone 145-2C11), murine CD4-PECy5 (clone GK1.5), murine CD8a-PECy5 (clone 53-6.7), murine CD117/cKit-PE (clone 2B8), murine

Sca1-PECy7 (clone D7), mB220-PECy7 (clone RA3-6B2), mGr1-PE (clone RB6-8C5), mCD11b-APC-eFluor780 (clone M1/70), murine Ter119-eFluor450 (clone TER-119), murine CD166/ALCAM-PE (clone ALC48), murine CD31-APC (clone 390), murine Lineage (CD3, B220, Gr1, CD11b, Ter119)-biotin from eBioscience (San Diego, CA, USA). Cells were incubated with respective antibodies and buffer (phosphate buffered saline with 0.5% bovine serum albumin) in 4°C in the dark for 15 minutes. Data were analyzed using FlowJo (Ashland, OR, USA).

Immunohistochemistry

Human and mouse tissues were fixed in 10% neutral-buffered formalin solution for maximum 48 h, dehydrated under standard conditions (Leica ASP300S, Wetzlar, Germany) and embedded in paraffin. BM specimen were decalcified in Osteosoft® (Merck Millipore, Darmstadt, Germany). Serial 2 µm thin sections prepared with a rotary microtome (HM355S, ThermoFisher Scientific, Waltham, USA) were collected and subjected to histological and immunohistochemical analysis. Hematoxylin-Eosin (H.-E.) staining was performed on deparaffinized sections with Eosin and Mayer's Haemalaun according to a standard protocol. For immunohistochemistry, slides were deparaffinized in xylene and rehydrated by alcohol washes of decreasing concentration (100%, 96%, 70%). After heat-induced antigen retrieval (target retrieval solution, pH 6 (Dako, Glostrup, Denmark, S1699)), unspecific protein and peroxidase binding was blocked with 3% hydrogen peroxide and 3% normal goat serum (Abcam, Cambridge, UK, 7481). Immunohistochemistry was performed with a Dako autostainer (Dako, Glostrup, Denmark) (CD3, CXCR4) or a Ventana Benchmark XT (Roche, Basel, Switzerland) (CD34) using antibodies against CD3 (clone SP7, DCS, Hamburg, Germany, CI597C01), CD34 (human specimen, (clone QBEnd/10, CellMarque, Darmstadt, Germany) and CXCR4 (clone UMB-2, Abcam, Cambridge, UK, 124824). For antibody detection, the Dako Envision-HRP rabbit labeled polymer (Dako, Glostrup, Denmark) or the UltraView Detection Kit (Roche, Basel, Switzerland) was used. Antibody binding was visualized by diaminobenzidine (DAB) giving a brown precipitate (Medac Diagnostica, Wedel, Germany, BS04-500). Counterstaining was performed using hematoxylin and slides were dehydrated by alcohol washes of increasing concentration (70%, 96%, 100%) and xylene and coverslipped using Pertex® mounting medium (Histolab, Goeteborg, Sweden, 00801).

Mice and patient-derived xenografts

For xenograft experiments, immuno-compromised NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice were purchased from Charles River (Charles River Laboratories Inc., Wilmington, MA, USA) and kept in a pathogen free environment in our animal facility. All experiments were approved by the regional authorities (Regierung von Oberbayern) in compliance with the German law on the protection of animals. Patient-derived xenograft models (PDX) were generated as previously described [50, 51].

Synthesis of Pentixafor and Pentixather

Synthesis of all used radiopharmaceuticals was performed in a fully automated, GMP-compliant procedure using a GRP® module (SCINTOMICS GmbH, Fürstenfeldbruck, Germany) equipped with disposable single-use cassette kits (ABX, Radeberg, Germany), using the method [28, 52] and standardized labeling sequence previously described [53]. Prior to injection, the quality of ^{68}Ga-Pentixafor was assessed according to the standards described in the European Pharmacopoeia for ^{68}Ga-Edotreotide (European Pharmacopoeia; Monograph 01/2013:2482; available at www.edqm.eu).

CXCR4 PET imaging in mice

PET imaging of leukemia-bearing mice was performed as previously described [23]. Briefly, mice were anesthetized with isoflurane and 12 MBq ^{68}Ga-Pentixafor was injected via the tail vein. After 75 min, static images were obtained for 15 min on a µPET-system (Inveon, Siemens, Erlangen, Germany).

Pentixather treatment *in vitro*

Whole bone marrow from healthy donors was cultured in DMEM (1 g/L Glucose) with 10% pooled human platelet lysate, Heparin (50 U/mL), 2 mM L-Glutamine, 1% Penicillin/Streptomycin to isolate plastic-adherent MSCs for in-vitro Pentixather treatment. Briefly, cells were treated for 10 min, 1 h and 6 h in 12-well plates with 1.5 MBq/mL Lu-P (comparable to 30 MBq distributed in a mouse weighing 20 g) or unlabeled Pentixather. For co-culture, CD34+ cells were isolated with CD34 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) via magnetic separation and ~1.5x10^4 cells were added to treated/control MSCs. After 4d of co-culture, flow cytometry and colony forming unit assays (StemMACS HSC-CFU complete with EPO, Miltenyi Biotec) were performed.

Pentixather treatment in mice

When leukemia was apparent in peripheral blood (4-5 weeks after injection), mice were subjected

to treatment with Lu-P or ctrl (unlabeled Pentixather) via tail vein injection, and then transferred to a radiation-restricted area for surveillance before they were sacrificed and relevant organs were harvested for further analysis. Mean injected radioactivity in ALL230, ALL0, OCI-AML3-eGFP and OCI-AML3-CXCR4 mice was 27.5 MBq, 23.4 MBq, 26.2 MBq and 25.2 MB1, respectively. Sample sizes were as follows: n=4 treated with Lu-P for 3d, n=4 treated for 7d with respective controls for ALL230, n=3 treated for 3d, n=4 treated for 7d with respective controls for ALL0, n=4 treated and n=4 control for AML346, n=3 treated and n=3 control for OCI-AML3-pHIV, n=4 treated and n=4 control for OCI-AML3-CXCR4.

Pentixather-based conditioning therapy in patients

Three patients with relapsed AML after alloSCT were referred for further therapy. Given the lack of alternative treatment options in this advanced disease stage, experimental CXCR4-directed treatment (with additional internal irradiation with ^{153}Sm-EDTMP or ^{188}Re-anti-CD66 antibodies for BM ablation) was offered on a compassionate use basis (German Drug Act, §13,2b) in compliance with §37 of the Declaration of Helsinki. Treatment was approved by the clinical ethics committee of our institution. All subjects gave written informed consent prior to therapy.

A pre-therapy dosimetry study using SPECT/CT and serial planar imaging was performed in patients scheduled for CXCR4-directed ERT after intravenous injection of ~200 MBq of Lu-P. This was done to i) record sites of unexpected tracer accumulation that may denote potential toxicity, ii) determine the organ radiation doses, and iii) to estimate the achievable tumor doses. The absorbed doses in tumors and organs were assessed by analyzing regions of interest in multiple planar total body images to obtain pharmacokinetic data and a single SPECT/CT scan to scale the pharmacokinetic curve.

All images were acquired using dual head gamma cameras (Siemens Symbia E for planar imaging, Siemens Symbia T2 calibrated from phantom measurements with ^{177}Lu activity standards for SPECT/CT acquisition) equipped with medium energy collimators. Pharmacokinetic data were fitted by bi-exponential functions. SPECT/CT data were reconstructed using a 3D-OSEM (6 subsets, 6 iterations, Gauss 6mm) algorithm with corrections for scatter and attenuation to obtain absolute activity quantification in voxels sized 0.11 cm^3. Estimates for the absorbed doses from treatment with Y-P were calculated from the 1 mL volumes with highest activity concentrations in dosimetry with Lu-P.

Based on their individual dosimetry, patients were treated by intravenous injection of ^{90}Y- labeled Pentixather. ERT was performed 4 and 7 days after pre-therapy dosimetry, respectively. To prevent renal toxicity, 2 L of a solution containing arginine and lysine (25 g/L each) was co-infused in analogy to the joint IAEA, EANM, and SNMMI practical guidance on peptide receptor radionuclide therapy in neuroendocrine tumors [54]. Vital signs, complete blood count, and blood chemistry were documented during the infusion and within 7 days after administration.

In order to enhance treatment effects, ^{90}Y-Pentixather therapy was followed by myeloablation by ^{153}Sm-EDTMP (patient #1) and ^{188}Re-anti-CD66-directed antibodies (patients #2 and #3).

Statistics

Statistics were performed with GraphPad Prism (GraphPad Software, La Jolla, CA). A two-tailed student's T-test was used to determine statistical significance (p value <0.05). Error bars represent mean ± standard error of the mean (SEM). Statistical significance is depicted as follows: * p<0.05, ** p<0.01, *** p<0.001.

Abbreviations

ALL: acute lymphoblastic leukemia; alloSCT: allogeneic hematopoietic stem cell transplantation; AML: acute myeloid leukemia; ATG: antithymocyte globulin; BFU-E: burst-forming unit, erythrocyte; BM: bone marrow; CFU: colony-forming unit; CFU-F: colony-forming unit fibroblast; CR: complete remission; ctrl: control; CXCR4: C-X-C chemokine receptor 4; EDTMP: ethylene diamine tetramethylene phosphonate; EMD: extramedullary disease; ERT: endoradiotherapy; EV: empty-vector control; iso: isotype antibody control; FLAG-IDA: fludarabine, cytarabine, G-CSF, idarubicine; FLAMSA-Bu-Cy-ATG: fludarabine, amsacrine, busulfane, cyclophosphamide, antithymocyte globulin; Flu-Bu: fludarabine, busulfane; G: granulocyte; HE: hematoxylin and eosin; GEMM: granulocyte, erythrocyte, monocyte, megakaryocyte; GM: granulocyte, monocyte; HSPC: hematopoietic stem and progenitor cells; ICE: idarubicine, cytarabine, etoposide; ID: initial diagnosis; ITD: internal tandem duplication; LSK: lineage negative, Sca1 positive, cKit positive stem cell; Lu-P: ^{177}Lu-Pentixather; M: monocyte; MFI: mean fluorescence intensity; MIP: maximum-intensity projection; MP: myeloid progenitor cell; MRD: minimal residual disease; MSC: mesenchymal stem cell; NSG: NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ; OBC: osteoblastic cell; PI: propidium

iodide; PB: peripheral blood; PDX: patient-derived xenograft; PET: positron emission tomography; PTD: partial tandem duplication; RR: relapsed/refractory; S-HAM: sequential high-dose cytarabine and mitoxantrone; Sp: spleen; TBI: total body irradiation; Y-P: ^{90}Y-Pentixather.

Supplementary Material

Table S1: Clinical characteristics of patients who provided primary material for PDX**. Table S2:** Characteristics of patients treated with Pentixather**. Fig. S1:** PDX establishment**. Fig. S2:** PDX surface markers. **Fig. S3:** P-AKT immunoblotting. **Fig. S4:** NSG control ^{68}Ga-Pentixafor imaging. **Fig. S5:** Lu-P in AML xenografts with enforced CXCR4 expression. **Fig. S6:** Lu-P in AML346. **Fig. S7:** Flow cytometry gating strategy. **Fig. S8:** Images of cultured stromal cells. **Fig. S9:** Lu-P treatment of primary human MSCs. **Fig. S10:** 68Ga-Pentixafor imaging and planar whole-body scintigraphic images in patients 1 and 2. http://www.thno.org/v08p0369s1.pdf

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Author contributions

S.H., C.L., P.H., U. G and U.K. designed the study and wrote the manuscript. C.L., A.K.B., A.S., H.H., and U.G. performed patient dosimetry and ERT. S.H., P.H., M.S., R.I., R.O. performed pre-clinical experiments, S.K. and S.K. performed histology and IHC. B.V. and I.J. established and provided PDX. K.G. provided patient data and interpretation and C.P. provided critical input. H.J.W. provided Pentixather and Pentixafor. All authors interpreted data, critically reviewed and approved the final manuscript.

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Competing Interests

H.J.W. is shareholder of Scintomics (Germany). All other authors have no relevant conflicts of interest to declare.

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Summary

Dual Targeting of Acute Leukemia and Supporting Niche by CXCR4-Directed Theranostics

Both refractory AML and ALL represent highly chemotherapy-resistant diseases and novel therapies to eliminate leukemic burden before allogeneic stem cell transplantation (alloSCT) are urgently needed. Based on the encouraging results of CXCR4-directed PET imaging (^{68}Ga-Pentixafor) in AML, we sought to further develop this concept to enable CXCR4-directed therapy ^{177}Lu-Pentixather in refractory acute leukemia. We applied CXCR4-directed theranostics to orthotopic patient derived xenograft (PDX) models of ALL and AML with different levels of CXCR4-expression and could demonstrate pronounced anti-leukemic activity of ^{177}Lu-Pentixather in these in-vivo models. We then performed more detailed analyses of the bone marrow hematopoietic niche by flow cytometry, immunohistochemistry and assays to evaluate functional damage to the niche and non-malignant hematopoietic cells and found that the niche is affected by Lu-Pentixather therapy, but is still capable of supporting healthy hematopoiesis. This finding was important for subsequent translation of CXCR4-directed theranostics into a clinical setting, because to fully eradicate leukemic cells, it is believed that targeting of the niche is beneficial, but excessive destruction of the microenvironment might compromise engraftment of donor stem cells and is to be avoided. We then incorporated Pentixather in the conditioning regimens of 3 patients with AML, who had relapsed after first alloSCT and had highly chemotherapy-refractory disease. In this limited cohort, Pentixather was well tolerated and donor stem cells engrafted within the expected time frame.

In conclusion, we showed that Pentixather efficiently kills AML and ALL cells in PDX models and that the bone marrow niche is targeted without abrogating the niche's ability to support healthy hematopoiesis after Pentixather treatment. First experience in highly treatment refractory patients with AML warrants further investigation of CXCR4-theranostics in clinical trials.

For this project, I performed and designed in-vitro and in-vivo experiments in PDX and cell line models (including flow cytometry, imaging, MSC experiments, colony forming unit assays, co-culture) and wrote the manuscript.

Discussion

Our work reveals that CXCR4-targeted imaging and therapy of patients with CXCR4-expressing acute leukemia is feasible and a promising concept worth following up further in clinical trials.

In the first study, we systematically assessed CXCR4 expression in AML and MDS patients treated at our institution and found detectable expression in a subset, consistent with existing literature (Rombouts, Pavic et al. 2004, Spoo, Lubbert et al. 2007, Mannelli, Cutini et al. 2014, Bae, Oh et al. 2015). To explore if the CXCR4 expression level on AML blasts is sufficient for imaging purposes with ^{68}Ga-Pentixafor, we performed ^{68}Ga-Pentixafor imaging in AML cell line xenografts with high, intermediate and low CXCR4 surface expression as determined by flow cytometry. Given the promising results, we further knocked out CXCR4 in a CXCR4-expressing AML cell line using CRISPR/Cas9 and could show that gene knock-out abolished PET-signal completely, which supports the ability of Pentixafor to bind to CXCR4 in a highly specific manner. Methodologically, this experiment also represents the first *in-vivo* imaging study using PET to demonstrate a gene knock-out with CRISPR/Cas9.

The series of 10 AML patients who underwent ^{68}Ga-Pentixafor PET imaging, 5 of which were CXCR4+ as determined by imaging, demonstrated that CXCR4-directed PET imaging is feasible in a subset of AML patients. However, this study comes with the limitation that the patient number was too low to draw conclusions about the value of CXCR4-PET in AML diagnostics. Importantly, the intention of evaluating CXCR4-directed PET imaging in AML was not to establish a new diagnostic standard, because a routine bone marrow biopsy or even a peripheral blood sample can detect malignant blasts with very high sensitivity and specificity already (Arber, Borowitz et al. 2017), but to establish a framework for therapeutic targeting using the therapeutic "partner" molecule of Pentixafor – Pentixather.

Our group and others have already shown the ability of ^{68}Ga-Pentixafor-based PET imaging to detect CXCR4-expressing lymphoma, multiple myeloma and several solid neoplasms (Philipp-Abbrederis, Herrmann et al. 2015, Wester, Keller et al. 2015, Vag, Gerngross et al. 2016). The application of ^{68}Ga-Pentixafor as a possibility to select patients for Pentixather-based therapy is particularly attractive in diseases with

bone marrow infiltration (leukemia, lymphoma, multiple myeloma), due to the expression of CXCR4 on HSPCs, which makes stem cell rescue necessary. In multiple myeloma, this approach has been performed in a limited number of highly pretreated patients and will be investigated further after promising initial results (Herrmann, Schottelius et al. 2016, Lapa, Herrmann et al. 2017). On the basis of these results, we designed a phase I/II study protocol to test CXCR4-theranostics in lymphoma and multiple myeloma followed by autologous stem cell transplantation in the COLPRIT study (Chemokine Receptor CXCR4-directed Theranostics of Advanced Lymphoproliferative Cancers by Radiopeptide-based Imaging and Therapy, EudraCT: 2015-001817-28), which received funding and is in planning to be initiated this year.

To further develop the novel therapeutic approach of CXCR4-theranostics, we focused on therapeutic targeting of AML and ALL cells in PDX models. These diseases are particularly suitable for CXCR4 theranostics for several reasons: When relapsed or resistant to chemotherapy, acute leukemias are extremely hard to treat and complete remissions are difficult to achieve, but essential for beneficial outcome, therefore novel, highly active therapeutic strategies are urgently needed (Dohner, Estey et al. 2017). Allogeneic stem cell transplantation remains the only curative option for most patients with relapsed/refractory disease, but toxicity of salvage and conditioning regimens limits its applicability to biologically fit patients. Because relapse is still the most common cause of death in AML and ALL, it is essential to eradicate all leukemic cells, especially treatment resistant LICs residing in protective niches; to reach sanctuary sites and due to its efficacy, total body irradiation is often used in conditioning before allogeneic stem cell transplantation, but is frequently not tolerated well (Hill-Kayser, Plastaras et al. 2011). With Pentixather, we wanted to explore a new approach to AML/ALL therapy and hypothesized that it is effective against leukemic blasts expressing CXCR4 and at the same time limits the radiation damage to the bone marrow and other sites of leukemic infiltration. We performed imaging and therapy with ^{177}Lu-Pentixather in 2 T-ALL PDX and 2 AML models and found that it efficiently reduces leukemic burden in infiltrated tissues. The use of xenograft models allowed us to analyze the cross-fire effect, i.e. the radiation-effect on cells that do not bind Pentixather but are in close proximity, because Pentixather binds only human CXCR4. We found that this cross-fire effect alone significantly

damages murine hematopoiesis, but at the same time does not affect MSCs in their role of supporting healthy hematopoietic cells, which is an essential prerequisite for engraftment of HSPCs during allogeneic stem cell transplantation.

Finally, 3 patients with relapsed AML after first allogeneic stem cell transplantation who would otherwise have received best supportive care due to the lack of treatment options were offered Pentixather-based salvage followed by a second allogeneic stem cell transplantation. What can be concluded from this case series is that i) ^{90}Y-Pentixather did not jeopardize engraftment of HSPCs, ii) no acute or delayed non-myeloablation-related toxicities attributable to ^{90}Y-Pentixather were observed and iii) 2 patients had full donor chimerism after allogeneic stem cell transplantation. Although the use of additional radiopharmaceuticals (^{153}Sm-EDTMP and ^{188}Re-anti-CD66) limits an estimate of the efficacy of Pentixather alone, the results are promising and warrant further investigation in a prospective clinical trial. In human patients, we chose ^{90}Y-Pentixather instead of ^{177}Lu-Pentixather due to its further reach to increase the bone marrow dose.

The immense potential of theranostic concepts has recently been demonstrated by a positive phase III trial of ^{177}Lu-Dotatate in neuroendocrine tumors (Strosberg, El-Haddad et al. 2017). In follicular lymphoma consolidation treatment with the anti-CD20 ^{90}Y-ibritumomab-tiuxetan showed prolongation of PFS, and was approved in this indication and for relapsed/refractor B-NHL (Morschhauser, Radford et al. 2013).

We believe that the concept of CXCR4-theranostics in acute leukemias is particularly suitable for AML and T-ALL, where it is evident that CXCR4 expressing cells contribute to disease progression, drug resistance and relapse (Zeng, Shi et al. 2009, Cho, Zeng et al. 2015, Passaro, Irigoyen et al. 2015). Current efforts to further refine this concepts include coupling Pentixather to alpha-emitters like Bismuth-213 or chemotherapeutic drugs like monomethyl auristatin E (the active compound in the approved antibody-drug conjugate brentuximab vedotin) that cannot be given systemically without being conjugated due to toxicity. An anti-CD33 Bismuth-213 conjugate and other agents conjugated to alpha emitters have produced promising initial results in leukemias and the higher linear energy transfer and lower reach of alpha emitters could increase specificity (Rosenblat, McDevitt et al. 2010, Scheinberg and McDevitt 2011).

In summary, we showed that i) CXCR4-directed imaging with ^{68}Ga-Pentixafor is feasible in AML patients and ii) a CXCR4-targeted theranostic concept with selection of suitable patients using PET and salvage therapy with Pentixather is effective in mouse models of acute leukemia and patients with relapsed/refractory AML without compromising engraftment of HSPCs. The manuscripts shown represent a truly translational approach with the ultimate goal to improve therapy of relapsed/refractory acute leukemias.

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