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. [68Ga]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. 1,3,5 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. 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. 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. 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. 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. As shown for several other cancers, CXCR4 expression negatively impacts prognosis in AML. Recent data in acute lymphoblastic leukemia (ALL) further substantiate the crucial role of this interaction in acute leukemia. 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 being developed in clinical trials, with encouraging results.

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. 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, 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 second-
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 CD45dim 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 classification34 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).

[68Ga]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 ([68Ga]), [68Ga]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 [68Ga]Pentixafor was feasible in vivo, we chose OCI-AML3 and NOMO-1 as CXCR4high and KG1a as CXCR4low cell line to generate subcutaneous xenograft mouse models. After tumor engraftment was apparent in all mice, [68Ga]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 [68Ga]Pentixafor in vivo.
To further test the specificity of \[^{68}\text{Ga}\]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. 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. \[^{68}\text{Ga}\]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. Thus, in vivo PET imaging of AML xenografts with \[^{68}\text{Ga}\]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 \[^{68}\text{Ga}\]Pentixafor to human CXCR4, 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.

Ten patients underwent \[^{68}\text{Ga}\]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 \[^{68}\text{Ga}\]Pentixafor-PET/MR and standard \[^{18}\text{F}\]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 \[^{68}\text{Ga}\]Pentixafor-PET. The PET positive areas correlated well with the expected signal...
alterations as determined by MR imaging (n=4, representative images shown in Figure 4A-B). 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 vertebrae 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 $[^{68}\text{Ga}]$Pentixafor-PET had biopsy-proven extramedullary relapse of AML after allogeneic stem cell transplantation (SCT) in the absence of BM involvement. $[^{68}\text{Ga}]$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 $[^{68}\text{Ga}]$Pentixafor-PET positive patients. Moreover, this CXCR4 positive lesion displayed high $[^{18}\text{F}]$FDG uptake (SUVmax 9.51) in the routine diagnostic $[^{18}\text{F}]$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 $[^{68}\text{Ga}]$Pentixafor-PET. Patient #10, who was visually negative in $[^{68}\text{Ga}]$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 $[^{68}\text{Ga}]$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.
The clinical significance of CXCR4 in AML is underscored by data showing that high CXCR4 expression on AML blasts correlates with poor prognosis. In a pediatric AML cohort, blast CXCR4 surface expression was increased by chemotherapy and contributed to resistance. There was no significant difference in CXCR4 surface expression between prognostic groups according to the modified ELN prognostic system 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. Such augmented interaction with the BM niche, in partic-

Figure 4. [18F]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 [18F]Pentixafor-PET/MR imaging. (G-I)[18F]Pentixafor-PET/MR images of a visually negative AML patient. (J-L) Control patient without BM malignancy who underwent [18F]Pentixafor-PET/MR imaging. (A, D, G, J) Maximum intensity projections of [18F]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 ilium; 4: lumbar 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 [18F]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).
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. 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. 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). 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. 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 99Yttrium and 177Lutetium, and compassionate use therapies have been applied to patients with very advanced multiple myeloma. 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 This, 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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