PIM Kinases Are Essential for Chronic Lymphocytic Leukemia Cell Survival (PIM2/3) and CXCR4-Mediated Microenvironmental Interactions (PIM1)

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Abstract

Overexpression of the CXCR4 receptor is a hallmark of chronic lymphocytic leukemia (CLL) and is important for CLL cell survival, migration, and interaction with their protective microenvironment. In acute myelogenous leukemia (AML), PIM1 was shown to regulate the surface expression of the CXCR4 receptor. Here, we show that PIM (proviral integration site for Moloney murine leukemia virus) kinases 1–3 are overexpressed and that the CXCR4 receptor is hyperphosphorylated on Ser339 in CLL compared with normal lymphocytes. Furthermore, CXCR4 phosphorylation correlates with PIM1 protein expression and PIM1 transcript levels in CLL. PIM kinase inhibition with three different PIM kinase inhibitors induced apoptosis in CLL cells independent of the presence of protective stromal cells. In addition, PIM inhibition caused dephosphorylation of the CXCR4 receptor on Ser339, resulting in enhanced ligand-dependent CXCR4 internalization and reduced re-externalization after withdrawal of CXCL12. Furthermore, PIM inhibition in CLL cells blocked CXCR4 functions, such as migration toward CXCL12- or CXCL12-induced extracellular signal–regulated kinase (ERK) phosphorylation. In concordance, pretreatment of CLL cells with PIM kinase inhibitors strongly reduced homing of CLL cells toward the bone marrow and the spleen of Rag2−/−;Rag1−/− mice in vivo. Interestingly, the knockdown of PIM kinases in CLL cells demonstrated diverging functions, with PIM1 regulating CXCR4 surface expression and PIM2 and PIM3 as important for the survival of CLL cells. Our results show that PIM kinase inhibitors are an effective therapeutic option for CLL, not only by impairing PIM2/3-mediated CLL cell survival, but also by blocking the PIM1/CXCR4-mediated interaction of CLL cells with their protective microenvironment. Mol Cancer Ther; 13(5); 1231–45. ©2014 AACR.

Introduction

B-cell chronic lymphocytic leukemia (B-CLL) is the most prevalent adult leukemia in Western countries and is characterized by a progressive accumulation of clonal CD5+ B lymphocytes in the peripheral blood, bone marrow, and lymphoid organs (1, 2). Several prognostic markers, such as the Rai and Binet staging systems (3, 4), immunoglobulin VH gene mutational status (5), ZAP70 expression (6, 7), and cytogenetic abnormalities (8) like Del 13q14, Del 17p, and Del 11q, can be used to predict the survival outcome in the Rai and Binet staging systems (3, 4), immunoglobulin VH gene mutational status (5), ZAP70 expression (6, 7), and cytogenetic abnormalities (8) like Del 13q14, Del 17p, and Del 11q, can be used to predict the survival outcome and direct treatment strategies for patients with CLL (9).

In addition to cell intrinsic alterations, microenvironmental factors are important for the survival of CLL cells. The cellular components of the CLL microenvironment are bone marrow stromal cells (BMSC), mesenchymal stromal cells in secondary lymphoid organs, monocyte-derived nurse-like cells, and T cells (10, 11). Coculture of CLL cells with BMSCs results in the spontaneous migration of a fraction of CLL cells beneath and underneath the BMSCs (12), protecting CLL cells from spontaneous apoptosis in vitro (13, 14), and inducing resistance to conventional chemotherapy in vivo (11, 15). One of the most important pathways mediating the interaction between BMSCs and CLL cells is the CXCR4/CXCL12 pathway (16). The G-protein–coupled receptor CXCR4 is highly expressed on the cell surface of CLL cells (12, 17), while BMSCs produce CXCL12 ligands, also known as stroma cell–derived factor...
CXCR4 activation by CXCL12 gradients induces CLL cell chemotaxis, migration across the vascular endothelium, actin polymerization, and migration under or underneath BMSCs and also shows direct antiapoptotic effects (12, 19, 20). Binding of CXCL12 to the CXCR4 receptor results in an internalization of the CXCR4 receptor by receptor endocytosis and pathway activation including extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) cascade activation and intracellular calcium flux (12).

PIM (proviral integration site for Moloney murine leukemia virus) kinases were first described as potential oncogenes by retroviral gene tagging in Moloney leukemia virus–induced lymphomas (21). Further studies revealed their proto-oncogenic activity in cooperation with other oncogenes such as c-MYC, N-MYC, or BCL2 (22). In mammals, three PIM serine/threonine kinases, PIM1, PIM2, and PIM3, are known (23). PIM kinases are constitutively active and are regulated predominantly by transcription through the JAK–STAT signaling pathway and proteasomal degradation (24, 25). PIM1 for example, is a direct target gene of STAT5 (26), which is frequently activated in hematologic malignancies. Overexpression of PIM kinases has been shown in various hematologic malignancies such as diffuse large B-cell lymphoma (27), CLL (PIM2; ref. 28), and FLT3-associated acute myelogenous leukemia (AML; ref. 29) as well as in some solid tumors (30).

PIM kinases regulate pro- and antiapoptotic members of the BCL2 protein family (31) and can block the proapoptotic protein BAD (32, 33). Furthermore, PIM1 has been shown to regulate cell-cycle regulators such as p21<sup>WAF1/CIP1</sup> (CDKN1A) and p27<sup>KIP1</sup> (34–36). Previous studies on AML have shown an interesting link between PIM kinase activity and the surface expression level and function of the CXCR4 receptor. Pim1 knockout mice displayed defects in hematopoietic stem cell (HSC) homing to the bone marrow and the spleen by downregulation of the CXCR4 surface expression. Furthermore, PIM kinase inhibition in primary AML blasts induced downregulation of the CXCR4 receptor on the cell surface and <i>PIM1</i> transcript levels correlated with CXCR4 surface expression in AML (37).

In the experiments shown here, we examined the role of PIM kinases and their effect on regulating the CXCR4 receptor in CLL. We found that the CXCR4 receptor is hyperphosphorylated on Ser339, and PIM1 and PIM2 are both overexpressed in the majority of CLL patient samples. PIM kinase inhibition induced apoptosis in CLL cells even in the presence of protective stromal cells. Furthermore, PIM kinase inhibition reduced CXCR4 surface expression, increased CXCL12-mediated internalization of the CXCR4 receptor, and abrogated CXCR4 functions, such as migration toward CXCL12 or extracellular signal-regulated kinase (ERK) phosphorylation, as well as the homing of CLL cells toward the bone marrow and spleen in vivo. Knockdown of PIM1–3 revealed diverging functions with PIM1 regulating CXCR4 surface expression and PIM2–3 as prosurvival genes.

### Materials and Methods

#### CLL patient samples

This study was approved by the Institutional Review Board of the University Medical Center Freiburg (Freiburg, Germany). Peripheral blood samples were obtained with informed consent in accordance with the Declaration of Helsinki from patients with B-CLL who were either untreated or off therapy for at least 6 months (summary of patient data and risk factors; Supplementary Table S1). CLL cases were characterized for IgV<sub>H</sub> mutational status, disease stage according to the Binet and Rai criteria, and history of treatment. Furthermore, genetic aberrations were analyzed by chromosomal analysis and FISH analysis and copy number changes were verified by single-nucleotide polymorphism (SNP) arrays (ref. 38; patient characteristics are shown in Supplementary Table S1). Peripheral blood mononuclear cells (PBMC) were separated by Ficoll gradient centrifugation and either used fresh or were cryopreserved in fetal calf serum (FCS) with 10% dimethyl sulfoxide (DMSO) until use. Cells were maintained in RPMI-1640 medium with 10% FCS and 1% penicillin–streptomycin. PBMCs for quantitative PCR (qPCR) or Western blot analysis contained more than 90% CD20<sup>+</sup> cells. Cells were lysed in RLT buffer for further RNA extraction or deep-frozen for protein extraction and subsequent immunoblotting.

#### Isolation of B cells from healthy donors

Whole blood samples from healthy donors were obtained from the blood tissue bank from the University of Freiburg (Freiburg, Germany). Mononuclear cells were isolated from whole blood samples by Ficoll gradient separation, followed by CD19 selection with anti-CD19 magnetic microbeads (Miltenyi Biotec) and the autoMacs Cell Separator (Miltenyi Biotec). Purity was determined by flow cytometry and was >90%. Cells were lysed in RLT buffer for further RNA extraction or deep-frozen for protein extraction and subsequent immunoblotting.

#### Cell lines

The murine BMSC line M2-10B4 (ATCC CRL-1972) and the human BMSC line HS-5 (ATCC CRL-11882) were both acquired from American Type Culture Collection (ATCC; 2010) and cultured in RPMI-1640 supplemented with 10% FBS. The cell lines were tested by ATCC and were authenticated in our laboratory by growth morphology and by the expression of laminin and collagen IV (M2-10B4) and of interleukin (IL)-6 and IL-8 (HS-5) by qPCR. MEC-1 (ACC-497), a human CLL cell line, was obtained from DSMZ (in 2012) and cultured in RPMI-1640 supplemented with 10% FBS and was authenticated by surface staining and flow cytometry for CD19, CD20, and CD79a.

#### Small-molecule inhibitors against PIM kinases and CXCR4 antagonists

The PIM kinase inhibitors K00135 and K00486 are imidazo[1,2-b]pyridazines and have been characterized...
Previously (34, 36). Both inhibitors show specific binding toward PIM1 and PIM2 (K00135 IC50 values: PIM1, 0.12 μmol/L; PIM2, 1.8 μmol/L; K00486 IC50 values: PIM1, 0.04 μmol/L; PIM2, 2.5 μmol/L) in vitro kinase assays. The small-molecule inhibitor A47 is based on another structural background, a furan thiazolidin (39). Besides good efficacy on PIM1 and PIM2, this inhibitor also blocks PIM3 kinase activity (IC50 values: PIM1, 3 μmol/L; PIM2, 0.8 μmol/L; PIM3, 2.6 μmol/L). The CXCR4 antagonistplerixafor (AMD3100; Sigma) is used in the clinic for mobilization of HSCs and for the treatment of CLL in early clinical trials (16).

**Quantitative real-time PCR**

Total cellular RNA was isolated from PBMCs from patients with CLL containing more than 90% of CD20+ CD5+ CLL cells and from CD19+ cells from healthy donors using the Qiagen RNAeasy Mini Kit. The cDNA was synthesized using 500 ng of each mRNA sample with Oligo-dT-Primers (Life Technologies), SuperScript II Reverse Transcriptase (Life Technologies), and deoxyribonucleotides (Fermentas) following the manufacturer's instructions. The mRNA transcript level was measured by quantitative TaqMan real-time PCR using a LightCycler 480 (Roche). TaqMan primers and probes were purchased from Applied Biosystems. The following primers were used: PIM1 hs00171473 m1, PIM2 hs00179139 m1, PIM3 hs00420511, CXCR4 hs00976734, and GAPDH 4310884E-090204. Results were quantified according to PIM3 m1,

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**Immunoblotting**

Protein expression of PIM1, PIM2, PIM3, and CXCR4 was determined in 11 human CLL samples and three normal CD19+ B lymphocyte (“NL”) samples using standardized protocols with antibodies against the following proteins: PIM1 (clone 12H8; Santa Cruz Biotechnology; 1:25), PIM2 (clone D1D2; Cell Signaling Technology; 1:500), PIM3 (clone RB8591; Abgent; 1:100), CXCR4 (clone 2074; Abcam; 1:500; 1:1,000), phospho-CXCR4 (pCXCR4; clone 74012; Abcam; 1:1,000), pERK (9106; Cell Signaling Technology; 1:1,000), ERK (9102; Cell Signaling Technology; 1:1,000), pBAD (5284; Cell Signaling Technology; 1:250), BAD (9239; Cell Signaling Technology; 1:250), and β-actin (clone AC-15; Sigma; 1:5,000). Western blot analyses were analyzed using the ImageJ software (NIH, Bethesda, MD).

**Apoptosis assay**

PBMCs were plated into 96-well plates at a concentration of 1 × 105 cells per well with or without support of the murine stromal cell line M2-10B4 (ATCC). In the case of stromal coculture, on day –1, 5 × 103 stromal cells per well were plated in 100 μL of medium containing RPMI-1640 with 10% FBS. After 24 hours, CLL cells were added and treated with three different PIM kinase inhibitors, the CXCR4 inhibitors AMD3100, or combinations at the indicated concentrations. After 24, 48, and 72 hours of incubation at 37°C in 5% CO2, cells were stained with a CD19-APC antibody (BD Biosciences), followed by Annexin V/7-AAD staining (BD Biosciences) according to the manufacturer’s instructions. Cells were analyzed using the CyAn ADP flow cytometer (Beckmann Coulter). Flow cytometry data were analyzed using the FlowJo 7.6 software (TreeStar).

**CXCR4 surface expression**

CLL cells were cultured in 96-well plates treated either with DMSO or the PIM kinase inhibitor 10 μmol/L K00135 for 2 hours. CLL cells from 96-well plates were stained with allophycocyanin (APC)-conjugated anti-human CD184 antibodies (clone 12G5; BD Biosciences) for CXCR4 surface expression and Annexin V/7-AAD (BD Biosciences) for analyzing apoptosis. Nonspecific binding was measured by APC-conjugated mouse IgG2a, κ (clone G155-178; BD Biosciences) as an isotype control.

**Intracellular phospho-ERK staining**

CLL cells alone or cocultured with M2-10B4 BMSCs were plated as above and treated with 10 μmol/L K00135, 100 μg/mL TN14003, or 20 μmol/L AMD3100. After 2 hours of incubation, cells were stained with CD19 antibody, fixed with 1.85% formalin, and permeabilized with 90% methanol. Endogenous levels of p44 and p42 MAPK were detected with the phospho-p44/42 MAPK mouse monoclonal antibody (#9106; Cell Signaling Technology) and anti-mouse immunoglobulin G (IgG) Alexa Fluor-conjugated antibody (#4408; Cell Signaling Technology). In the case of CXCL12 stimulation, cells were treated with 500 ng/mL of CXCL12 (R&D Systems) for 30 seconds before fixation. Cells and data were analyzed as described above.

**Chemotaxis assays**

CLL cells were preincubated for 2 or 24 hours with 10 μmol/L of PIM inhibitor K00135 in a suspension of RPMI-1640 and 0.5% bovine serum albumin. A volume of 100 μL containing 5 × 10^6 CLL cells was added to the top chamber of a 6.5-mm diameter Transfer culture insert (Coster) with a pore size of 5 μm. Filters were then transferred to medium either with or without 500 ng/mL of CXCL12. Chambers were incubated for 2 or 24 hours at 37°C and 5% CO2. Then, the lower chamber was suspended and divided into aliquots for counting with the CyAn ADP flow cytometer (Beckmann Coulter) for 40 seconds at 100 μL/min.

**CXCR4 recycling assay**

CLL cells were seeded into 96-well plates with and without 500 ng/mL CXCL12 and treated with DMSO or
10 µmol/L K00135 for 12 hours, followed by three washing steps with PBS to remove CXCL12 and K00135 and resuspension in medium. After treatment and 12 hours after washing out CXCL12 and K00135, the surface of CLL cells was stained with APC-conjugated anti-human CD184 antibody. Then, cells were fixed with 1.8% formalin and permeabilized with 90% methanol. Total (cytoplasmic and membrane bound) levels of CXCR4 were detected with primary anti-human CD184 antibody (2074; Abcam; 1:100) and with secondary antibody Alexa Fluor 488 goat anti-rabbit (Life Technologies, 1:100). For nuclear staining, slides were incubated with 4′,6-diamidino-2-phenylindole (DAPI; Sigma; 1:1,000). CLL cells were collected for flow cytometry, immunofluorescence analysis using confocal microscopy (Leica SP2 AOBS spectral confocal microscope), and High Content Screening using the Olympus ScanR.

**CLL cell homing into Rag2⁻/⁻ /C0 mice**

Rag2⁻/⁻ /C0 mice were bred and handled under sterile conditions and transplanted at 8 to 10 weeks of age after sublethal irradiation (3.0 Gy) 18 hours before transplantation. Primary human CLL cells were stained with 1 µmol/L carboxyfluorescin diacetate succinimidyl ester (CFSE; Life Technologies) according to the manufacturer’s instructions and treated with 10 µmol/L K00135 for 12 hours. Viability of the cells was assessed by Annexin V/7-AAD staining. Subsequently, CLL cells were resuspended in Hank’s Balanced Salt Solution (HBSS) and irradiated mice received transplants of 3.5 × 10⁷ PBMCs via tail vein injection. Mice were analyzed 4 hours after transplantation. Single-cell suspensions of spleens and bone marrow were stained with APC-labeled anti-human CD45 antibody (BD Biosciences) and PE-labeled anti-human CD19 antibody (BD Biosciences) and CD45⁺/CD19⁻/CFSE cells were detected by flow cytometry.

All animal experiments were approved by the Regierungspädisäum Freiburg and were in accordance with the U.S. NIH Statement of Compliance with Standards for Humane Care and Use of Laboratory Animals.

**siRNA-mediated PIM1–3 knockdown**

Freshly isolated CLL cells at a concentration of 1 × 10⁷ were resuspended in Nucleofection solution (Nucleofector KitV; Lonza). siRNAs for PIM kinases were added at a concentration of 20 µmol/L (PIM1: Si00040978, Si00040985, and Si02629165; PIM2: Si00092869 and Si02224201; PIM3: Si00684915 and Si03084543; Qiagen). Transfection was started using program U-13 of Amaxa Biosystems Nucleofector II (Amaxa Biosystems). After 24 hours, one part of the cells was stained with CD184-APC and Annexin V-PE/7-AAD (both BD Biosciences) and analyzed as described above. The other part of the cells was used for immunoblotting for PIM kinases 1–3, phospho-BAD, BAD, and β-actin. Knockdown (KD) efficiency was calculated relative to β-actin using ImageJ software and compared with NoSi control.

**Statistical analysis**

Data are represented as the mean ± SEM. Comparisons between parameters were performed using a two-tailed, paired Student t test or the Mann–Whitney test. Correlations were assessed with the Spearman correlation coefficient. For all analyses, P < 0.05 was considered statistically significant.

**Results**

**PIM1 and PIM2 are overexpressed and the CXCR4 receptor is hyperphosphorylated on Ser339 in CLL**

Previous studies have shown an increase in the expression of the PIM2 kinase in CLL cells by using qPCR and intracellular fluorescence-activated cell sorting (FACS) staining [Cohen and colleagues (28); and Chen and colleagues, (41)]. To verify these results in our CLL cohort (Supplementary Table S1), we investigated the expression level of all three PIM kinases at the transcriptional and protein levels. Using quantitative real-time PCR (qRT-PCR), we identified a significant increase in PIM2 (P = 0.0404), but also in PIM1 (P = 0.0060) transcript levels in CLL cells (n = 15) compared with normal B lymphocytes (n = 4) from healthy donors (Fig. 1A). Median PIM1 levels were increased 5.1-fold and median PIM2 levels were increased 1.7-fold. There was a tendency toward higher RNA expression levels of PIM3 and CXCR4 in CLL cells compared with normal B lymphocytes, which was not statistically significant (Fig. 1A).

To evaluate whether or not elevated transcript levels are translated into increased expression of PIM kinases at the protein level, we performed Western blot analyses for PIM1, PIM2, PIM3, CXCR4, and pCXCR4 with B-CLL lymphocytes (n = 11) and normal lymphocytes from healthy donors (n = 3). Also on protein levels, PIM1 and PIM2 were significantly overexpressed in CLL samples compared with normal B lymphocytes and relative to β-actin (PIM1, P = 0.0142; PIM2, P = 0.0251; Fig. 1B and C). Relative levels of PIM1 protein were increased 2.5-fold with increased protein levels in 10 of 11 CLL samples, while relative levels of PIM2 were increased 6-fold with increased protein levels in 11 of 11 CLLs (Fig. 1C). Interestingly, the smaller isoform of PIM1 at 37 kD was present only in CLL cells, but not in normal B lymphocytes. Relative levels of PIM3 protein were increased in six of 11 CLL samples (Fig. 1B and C). There was no correlation in between PIM transcript levels or PIM protein expression levels and risk factors in CLL, like stage of disease or mutational status.

Previous studies on HSCs have shown that PIM1 can phosphorylate Serine339 on the CXCR4 receptor and that this regulates CXCR4 surface expression (37). In CLL, it is known that the CXCR4 receptor is highly upregulated on the cell surface compared with normal B lymphocytes, while total CXCR4 protein levels are normal. As previously described, total CXCR4 RNA and protein levels were not significantly elevated in our CLL cohort compared with normal B cells (Fig. 1A–C). In contrast, CXCR4...
Figure 1. PIM1/2 and pCXCR4 are overexpressed in CLL. A, expression of PIM1, PIM2, PIM3, and CXCR4 mRNA in B-CLL cells (CLL) versus normal lymphocytes (NL) from healthy donors by qRT-PCR analysis. The values are normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript levels and represent each CLL patient or normal lymphocytes donor. Mean value for controls is set to one. Experiments were performed in independent duplicates or triplicates (Mann–Whitney test). Transcript levels for PIM1 and PIM2 are elevated in CLL patient samples compared with controls (PIM1, \( P = 0.0060 \); PIM2, \( P = 0.0404 \); patient samples CLL 1–16 from Supplementary Table S1). B, protein expression of pCXCR4, CXCR4, PIM1, PIM2, and PIM3 in B-CLL lymphocytes (CLL#) from patients with CLL and normal lymphocytes from healthy donors analyzed by Western blot analysis and using β-actin as a loading control. C, protein expression levels were quantified using the ImageJ software (NIH), and the expression levels of pCXCR4, CXCR4, PIM1, PIM2, and PIM3 were normalized to β-actin expression levels. Mean expression levels of specific genes compared with β-actin in normal lymphocytes were set to 1. PIM1 and PIM2 protein levels and CXCR4 phosphorylation is significantly elevated in CLLs compared with normal lymphocytes (PIM1, 2.5-fold increase, \( P = 0.0124 \); PIM2, 6-fold increase, \( P = 0.0251 \); pCXCR4 with 4.6-fold increase). Total levels of PIM3 and CXCR4 are not significantly altered in CLLs compared with normal lymphocytes. D, Spearman correlation coefficient of pCXCR4 protein and PIM1 protein level (\( r = 0.6088; P = 0.0209 \)).
phosphorylation on Ser39 was strongly increased in CLls compared with normal lymphocytes (Fig. 1B and C; 4.6-fold increase). There was a direct positive correlation in between CXCR4 phosphorylation and PIM1 kinase protein expression in CLL cells (Spearman correlation coefficient, P = 0.0209; Fig. 1D).

As previously described, CXCR4 surface expression levels were significantly elevated in our CLL cohort (Fig. 2A). PIM1 kinase expression on transcriptional level and also on protein level both positively correlated with the surface expression of the CXCR4 receptor (Fig. 2B and C), pointing toward PIM1 as a potential regulator of CXCR4 surface expression in CLL.

**PIM kinase inhibition with three different PIM inhibitors induces apoptosis in CLls independent of coculture with stromal cells**

To verify the importance of PIM kinase activity for the survival of CLL cells, we investigated the potential of three different small-molecule PIM kinase inhibitors on apoptosis induction in CLL cells (34, 36, 39). K00135 and K00486, two imidazo[1,2-three different small-molecule PIM kinase inhibitors on survival of CLL cells, we investigated the potential of coculture with stromal cells. PIM inhibitors for 48 hours showed a dose-dependent increase in apoptosis (Fig. 3A for K00135, for example, CLL 10) and a significant reduction in viable cells (Fig. 3B). PIM1/PIM2 kinase inhibitors K00135 and K00486 at 10 μmol/L reduced the mean cell viability to about 50% after 48 hours of incubation (Fig. 3C). Treatment with the A47 PIM1–3 kinase inhibitor also showed a significant induction of apoptosis, although its efficacy was lower than that of the two other compounds.

Previous studies have demonstrated the importance of the microenvironment, and especially the presence of stromal cells, for the survival of CLL cells (10, 42). The interaction between BMSCs and CLL cells induces a protective microenvironment blocking the proapoptotic effect of various chemotherapeutical agents and spontaneous apoptosis in vitro. Pathways involved in this process are the CXCL12–CXCR4 axis, Hedgehog (HH) ligands activating the HH signaling pathway in CLL cells, and various other chemokines and integrins (12, 16, 43). To evaluate the effect of protective stromal cells on the effect of PIM kinase inhibitors in CLL, we cocultured CLL cells with the BMSC line M2-10B4 (baseline viability with and without stroma is shown in Supplementary Fig. S1). Interestingly, M2-10B4 cells could not sufficiently protect the CLL cells from PIM inhibitor–induced apoptosis, although the effect of the PIM inhibitors was stronger in the absence of stromal cells (Fig. 3C). Mean IC₅₀ values without stroma were lowest for K00486 (2.40 μmol/L) compared with K00135 (2.96 μmol/L) and A47 (4.10 μmol/L). The presence of stromal cells significantly increased the IC₅₀ values for K00135 (mean IC₅₀, 4.61 μmol/L; P = 0.0029) and A47 (mean IC₅₀, 5.71 μmol/L; P = 0.0148; Fig. 3D), but not for K00486 (mean IC₅₀, 3.36 μmol/L; P = 0.0571; Fig. 3D).

Two possible mechanisms could be responsible for this low protective effect. Either PIM inhibitors can directly block the protective pathways coming from the stromal cells, or the stromal cells are directly affected by the PIM inhibitors.

To address both possibilities, we first investigated the presence of the PIM–CXCR4 signaling pathway in the murine stromal cell line M2-10B4 (MS) and in the human stromal cell line HS-5 (HS). Interestingly, besides CLL cells, also BMSCs express all three PIM kinases and the CXCR4 receptor (Fig. 4A). High PIM inhibitor concentrations of K00486 and K00135 (10 μmol/L) could induce apoptosis in murine stromal cells with IC₅₀ values between 8 and 10 μmol/L (stroma IC₅₀ values for K00135, 8.2 μmol/L; K00486, 9.65 μmol/L; and A47, >10 μmol/L; Fig. 3B and D), while there was no significant apoptosis induction in M2-10B4 cells after treatment with the A47 inhibitor.
To investigate whether PIM kinases and the important CXCR4-CXCL12 axis share the same survival signals, we investigated the effect of a combination of the PIM kinase inhibitor K00135 and the potent CXCR4 inhibitor plerixafor on CLL cells (n = 5) in the presence of M2-10B4 BMSCs. Treatment of CLL cells with 20 μmol/L plerixafor could significantly induce moderate apoptosis in CLL cells (Fig. 4B). The treatment of CLL cells with 5 μmol/L K00135 showed a significant and strong induction of apoptosis as measured by Annexin V/7-AAD staining after 48 hours. The addition of plerixafor to the PIM inhibitor K00135 could not enhance apoptosis induction, which indicates that proapoptotic effects induced by plerixafor might already be blocked by the PIM kinase inhibitor (Fig. 4B). Taken together, the lack of CLL protection by stromal cells might be caused by a dual effect of PIM inhibitors, with directly affecting stromal cell viability at least at high inhibitor
concentrations on the one hand, and direct interference with CLL survival pathways activated by stromal cells, like CXCR4, on the other.

**PIM kinase inhibitors reduce CXCR4 surface expression and CXCR4 phosphorylation in CLL**

To further investigate the second possibility, we investigated the effect of PIM inhibitors on CXCR4 surface expression and CXCR4 phosphorylation. In AML, Grundler and colleagues identified PIM1 as a regulator for the surface expression of the CXCR4 receptor (37). Treatment of CLL cells with the K00135 PIM kinase inhibitor resulted in a significant decrease in CXCR4 surface expression levels \((n = 5)\), indicating that PIM kinases can act as regulators for CXCR4 surface expression also in CLL (Fig. 5A). In contrast to the downregulation of CXCR4 surface expression by PIM inhibition, we found that the CXCR4 mRNA levels were upregulated in the same samples after PIM inhibition, which suggests a counteracting regulatory loop in CLL cells after downregulation of CXCR4 surface expression (Fig. 5B).

Grundler and colleagues previously identified phosphorylation of Ser339 to be critical for CXCR4 surface expression on AML cells (37). Therefore, we also investigated the phosphorylation level of Ser339 in CLL cells treated with PIM kinase inhibitors and found a strong reduction of Ser339 phosphorylation within 6 hours of treatment (Fig. 5C).

**PIM kinase inhibition enhances CXCL12-mediated internalization of the CXCR4 receptor in CLL cells**

To further characterize the effect of PIM kinase inhibition on the surface expression of the CXCR4 receptor, CLL cells (four independent experiments with four different...
patients with CLL) were incubated for 12 hours with 500 ng/mL of CXCL12 to induce internalization of the CXCR4 receptor and were treated with either DMSO or 10 μmol/L K00135. Viability of cells as assessed by Annexin V/7-AAD staining after this treatment was more than 90% in both treatment groups (data not shown). CLL cells treated with CXCL12 showed a nearly complete down-regulation of CXCR4 from the cell surface, as shown by flow cytometry (Fig. 6A, second lane) and by dual color confocal microscopy (APC-labeled CXCR4 membrane staining in red and Alexa Fluor 488–labeled intracellular CXCR4 staining in green; Fig. 6B, mid lane). Cells treated with the PIM inhibitor K00135 showed a slight decrease in baseline CXCR4 surface expression (Fig. 6A) as measured by flow cytometry. Upon treatment with CXCL12, CLL cells with PIM inhibition also showed CXCR4 internalization (Fig. 6A and B) and showed clustering of the CXCR4 receptor in the cytoplasm (Fig. 6B). In the control group, washout of CXCL12 for 12 hours resulted in a slow, but measurable re-externalization of the CXCR4 receptor to the cell surface (Fig. 6A–D), whereas CLL cells with previous PIM kinase inhibition showed a further decrease in surface CXCR4 levels and accumulation of CXCR4 in clusters in the cytoplasm (Fig. 6B). Flow cytometry data were quantitated as mean fluorescence intensity (MFI) in Fig. 6C and for a CLL cell line (MEC-1) in Supplementary Fig. S2. In addition to the confocal microscopy, we also performed high-resolution imaging and quantitated cells with mainly membrane staining (red ring) versus cells with mainly intracellular staining (green). By using this method, we clearly see the internalization of the CXCR4 receptor after CXCL12 stimulation (Fig. 6D). Furthermore, we can detect re-externalization of the CXCR4 receptor to the membrane after CXCL12 wash-out, which is totally absent after PIM kinase inhibitor treatment (Fig. 6D).

Taken together, these results indicate an important regulatory role for PIM kinases on CXCR4 surface expression, and especially the re-externalization of the receptor in CLL.
PIM kinase inhibitors block the function of the CXCR4 receptor regarding migration and ERK phosphorylation

Previous studies have shown that CXCR4-expressing cells can migrate toward a CXCL12 gradient (12). To verify that PIM kinases can not only affect the surface expression of the CXCR4 receptor, but can also affect the function of CXCR4, we performed migration assays of CLL cells toward a CXCL12 gradient and without CXCL12 (right) or M2-10B4 stromal cells and treated with PIM inhibitor K00135 (K) or the CXCR4 inhibitor AMD3100 (A) for 3 or 6 hours (CLL#13). C, MFI for phospho-ERK measured by flow cytometry in CLL cells cultivated with or without CXCL12 or M2-10B4 stromal cells and treated with the PIM inhibitor K00135 or the CXCR4 inhibitor AMD3100 (n = 7; paired t test; analyzed patients top left, top right, and bottom left: 4, 13, 15, 17, 19, 20, and 21; bottom right: 4, 13, 15, 17, 18, 20, and 21). D, Western blot analysis for phospho-ERK and ERK after 3 hours and for phospho-BAD, BAD, pCXCR4, and CXCR4 after 24 hours of treatment with PIM inhibitors K00486 and A47 (CLL#19).

To further examine the effect of PIM kinase inhibition on CXCR4 receptor signaling, we investigated the effect of PIM inhibition on the MAPK pathway, which is a known CXCR4 downstream signaling pathway. Stimulation of chamber (CLL#16, 3.8% after 2 hours and 30% after 24 hours; Fig. 7A), yet this effect was nearly completely abolished upon PIM kinase inhibition (with PIM inhibitor, 2.2% after 2 hours and 3% after 24 hours; Fig. 7A). Three other CLL samples showed very similar results (mean of migrating cells, DMSO 41% and K00135 15%), indicating a functional loss of the CXCR4 receptor upon treatment with PIM kinase inhibitors (Fig. 7A, third panel).
PIM Kinases Regulate CLL Survival and CXCR4 Surface Levels

Figure 8. PIM kinase inhibition reduces migration of CLL cells into the spleen and bone marrow of Rag2<sup>−/−</sup>/γc<sup>−/−</sup> mice. CLL cells with recombinant CXCL12 or coculture of CLL cells with CXCL12-producing stromal cells (M2-10B4) induced ERK phosphorylation as shown by intracellular phospho-flow cytometry and Western blot analysis (Fig. 7B and C). Treatment of CXCL12-stimulated CLL cells with the CXCR4 inhibitor AMD3100 (plerixafor) strongly inhibited ERK phosphorylation as shown by intracellular phospho-flow cytometry and Western blot analysis (Fig. 7B and C). Interestingly, PIM kinase inhibition with 10 μmol/L K00135 had the same effect on ERK phosphorylation, indicating an interference with the CXCL12–CXCR4 signaling pathway in CLL cells (Fig. 7B and C). In addition, ERK phosphorylation in CLL cells induced by coculture with CXCL12-producing M2-10B4 stromal cells could be abrogated by CXCR4 inhibitors or PIM inhibitors (Fig. 7B).

Also the other two PIM kinase inhibitors (K00486 and A47) were effective in reducing ERK phosphorylation even in the absence of CXCL12 stimulation (Fig. 7D) and could reduce phosphorylation of the known PIM kinase targets BAD and the CXCR4 receptor (Fig. 7D).

**PIM kinase inhibition in CLL cells blocks migration of CLL cells into the bone marrow and spleens of Rag2<sup>−/−</sup>/γc<sup>−/−</sup> mice**

The CXCR4 receptor is known to be important for the homing of mature B cells and CLL cells into the bone marrow and spleens of xenotransplanted mice. To investigate whether the observed internalization of the CXCR4 receptor induced by PIM inhibition is also functional in vivo, we pretreated CFSE-stained CLL cells with the PIM kinase inhibitor K00135 (10 μmol/L) or with DMSO for 12 hours. Then, CLL cells were collected for in vitro experiments and some cells were separated to assess apoptosis. Apoptosis was measured using Annexin V/7-AAD staining and cell viability was greater than 90% for both groups (Supplementary Fig. S3). Four irradiated Rag2<sup>−/−</sup>/γc<sup>−/−</sup> mice per group were each transplanted with 3.5 × 10<sup>7</sup> CLL cells via tail vein injection. After 4 hours, the mice were sacrificed and the homing of CLL cells into spleens and bone marrow was assessed by flow cytometry. CLL cells that were pretreated with the PIM inhibitor showed a significant decrease in their homing capacity for both organs (Fig. 8). The amount of CLL cells (CD45<sup>+</sup>CFSE<sup>−</sup>CD19<sup>+</sup>) in the bone marrow was 2.3% in the control group versus 1% in the treatment group (P = 0.0293) and in the spleen 23.3% versus 9.3%, respectively (P = 0.0104; Fig. 8), indicating that interference with the CXCR4 receptor function also occurs in vivo.

**siRNA knockdown of PIM1–3 in CLLs shows diverging functions regarding regulation of CXCR4 and apoptosis induction**

To further dissect the PIM kinase–dependent functions regarding apoptosis induction and CXCR4 receptor regulation, we performed siRNA knockdown with two to three siRNAs per PIM kinase in five different CLL samples using the Ammaxa technology as previously described (Fig. 9A; refs. 44, 45). siRNA knockdown of PIM1 induced a significant downregulation of the CXCR4 surface expression with three different siRNAs targeting PIM1, but did not induce apoptosis in CLL cells (Fig. 9B and C). In contrast, PIM2 knockdown did not significantly alter the CXCR4 surface expression, but could induce apoptosis in CLL cells. PIM3 knockdown had the strongest proapoptotic effect in CLL cells, but did not alter the CXCR4 surface expression (Fig. 9B and C). To further dissect the diverging functions of PIM kinases, we also investigated the effect of the PIM knockdown on BAD phosphorylation and total BAD protein levels. Interestingly, while PIM1 did not alter BAD phosphorylation levels, both PIM2 and PIM3 reduced BAD phosphorylation and total BAD levels after PIM2 knockdown (Fig. 9D), which might explain the proapoptotic effects seen after knockdown of PIM2 and PIM3 (32, 46–48).
Taken together, the proapoptotic effects of PIM inhibitors and their effect on CXCR4 downregulation from the cell surface are mediated by different PIM kinases, with PIM1 regulating the CXCR4 surface expression and PIM2/PIM3 supporting survival of CLL cells.

Discussion

The CXCR4 receptor is crucial for homing, migration, and retention of various hematopoietic cell types, such as HSCs, B cells, and also CLL cells, into the bone marrow (12, 49). Furthermore, CXCR4 signaling provides direct prosurvival signals and protects CLL cells from the effect of chemotherapeutical agents in vivo (11, 15). The function of the CXCR4 receptor is strongly dependent on its localization on the cell surface and is activated upon ligand binding of CXCL12 (16). Therefore, current efforts are focused on direct CXCR4 inhibitors (TN14003, plerixafor), blocking the interaction of the CXCR4 receptor with the CXCL12 ligand (50, 51). Plerixafor is currently used for the mobilization of HSCs from the bone marrow and is tested in ongoing clinical trials for its effect on mobilizing CLL cells for chemosensitization away from their protective microenvironment (52). Unfortunately, the use of CXCR4 inhibitors induces a counter-regulatory upregulation of CXCR4 on the cell surface of the target cells, resulting in a short-lived (8–12 hours) and only partial mobilization of CXCR4-expressing cells into the peripheral blood. In contrast to CXCR4 antagonists blocking CXCR4 signaling, PIM inhibitors block the externalization of the CXCR4 receptor and might therefore be able to circumvent the counter-regulatory upregulation of CXCR4 upon plerixafor treatment. Our data suggest that at least various aspects of CXCR4 signaling in CLL cells can be affected by PIM kinase inhibition. PIM kinase inhibition strongly affected the surface expression of the CXCR4 receptor on CLL cells, and induced dephosphorylation of the Ser339 residue in the C-terminal tail of the receptor, resulting in reduced recycling of the CXCR4 receptor to the cell surface. Furthermore, PIM kinase inhibition was able to effectively block various functions of the CXCR4 receptor, such as migration toward CXCL12, CXCL12-mediated ERK phosphorylation, and even in vivo homing of CLL cells into the bone marrow and the spleens of Rag2−/− γc−/− mice. Therefore, single use of PIM inhibitors might already be sufficient for CLL-cell mobilization or could be an intelligent addition to the plerixafor treatment to avoid counter-regulatory CXCR4 upregulation. Treatment studies combining plerixafor and PIM inhibitors in CLL xenograft mouse models will be performed to proof this concept in vivo.

In concordance with those findings, the presence of protective stromal cells could not efficiently block apoptosis of CLL cells treated with PIM inhibitors. By using...
the PIM inhibitor K00135, we observed apoptosis rates higher than 50% in CLL cells that were cultivated alone. Interestingly, PIM inhibition in CLL cells cocultivated with protective stromal cells showed similar rates of apoptosis, indicating that the stromal protection, which is at least partially mediated by CXCL12/CXCR4, is not functional in the presence of PIM inhibitors. As stromal cells have an intact PIM-CXCR4 axis, they can be directly affected by PIM kinase inhibitor treatment. Although the apoptosis induced in M2-10B4 stromal cells only occurred at very high inhibitor concentrations, we cannot exclude that the secretion of cytokines/integrins is already affected at lower concentrations and might therefore indirectly reduce the stroma-protective effect on CLL cells.

The role of the three Pim kinases for normal and malignant hematopoiesis shows strongly overlapping functions at least in various murine lymphoma models. Pim3 was able to compensate for the loss of Pim1 and Pim2 in MuLV-induced lymphomagenesis (23). Furthermore, Eμ-Myc-Eμ-Pim1 double-transgenic mice showed enhanced tumor development similar to that seen in Eμ-Myc-Eμ-Pim2 transgenic mice, pointing toward redundant functions of PIM kinases in Myc-dependent B-cell lymphoma development (22, 53).

Previous work on PIM kinases in CLL has identified PIM2 as a proapoptotic target in CLL regulating C-MYC and MCL-1 (41), while PIM1 and PIM3 or the interaction of PIM kinases with the CXCR4 receptor were not investigated. As previously described (28), we could confirm overexpression of PIM2 in our patient cohort, but also found strong overexpression of PIM1 in most CLLs and of PIM3 in a portion of more than 50% of CLL samples as measured by qPCR and Western blot analysis. As several PIM kinases are overexpressed in CLL, we wanted to dissect their functions by using siRNA knockdown of each PIM kinase in primary CLL samples. Interestingly, only knockdown of PIM1 could significantly alter surface expression of the CXCR4 receptor in CLLs, while PIM3 and PIM2 were not able to affect CXCR4 surface expression in CLL. In line with those findings, only PIM1 protein levels correlated with pCXCR4 protein levels and PIM1 transcript and protein levels positively correlated with CXCR4 surface expression levels. Therefore, high PIM1 expression might contribute to increased CXCR4 surface expression identified on CLL cells, and can affect the interaction of CLL cells with their microenvironment.

On the other hand, knockdown of PIM3 and to a lower degree also of PIM2 induced apoptosis in CLL cells, which could not be observed upon PIM1 knockdown. In line with those findings, PIM2 and PIM3 knockdown could reduce phospho-BAD levels and total BAD in CLL cells, which was not observed after PIM1 knockdown. Those results strongly argue for nonredundant functions of those three PIM kinases in CLL and separate survival from CXCR4 surface expression. Consequently, pan-PIM inhibitors should be developed for the treatment of CLL, to block PIM1/CXCR4–dependent CLL cell migration and to induce PIM2/3–dependent apoptosis.

In general, our results show that PIM kinase inhibitors are an effective treatment option for CLL not only by directly inducing proapoptotic pathways in CLL cells, but also by blocking the interaction of CLL cells with their protective microenvironment through the CXCR4 receptor.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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