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Cross-Talk between CLL Cells and Bone Marrow Endothelial Cells: Role of Signal Transducer and Activator of Transcription-3

Xavier Badoux, MD^a, Carlos Bueso-Ramos, MD, PhD^b, David Harris, BS^a, Ping Li, PhD^a, Zhiming Liu^a, Jan Burger, MD^a, Susan O'Brien, MD^a, Alessandra Ferrajoli, MD^a, Michael J. Keating, MD^a, and Zeev Estrov, MD^a

^aDepartment of Leukemia, The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030

^bDepartment of Hematopathology, The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030

Summary

Chronic lymphocytic leukemia (CLL) bone marrow is characterized by increased angiogenesis. However, the molecular mediators of neovascularization and the biological significance of increased endothelial cell proliferation in CLL require further investigation. Because signal transducer and activator of transcription (STAT)-3 is constitutively activated in CLL we studied the role of STAT3 in modulating vascular endothelial growth factor (VEGF) expression and the effect of vascular endothelial cells on CLL cells. Using chromatin immunoprecipitation (ChIP) we found that anti-STAT3 antibodies immunoprecipitated DNA of STAT3, VEGF and other STAT3-regulated genes. In addition, STAT3-short interfering RNA significantly reduced mRNA levels of VEGF in CLL cells suggesting that STAT3 induces VEGF expression in CLL. Remarkably, bone marrow CLL cells expressed high levels of VEGF and high VEGF levels were detected in the plasma of patients with untreated CLL and correlated with white blood cell count. CLL bone marrow biopsies revealed increased microvascular density and attachment of CLL cells to endothelial cells. Co-culture of CLL and human umbilical vein endothelial cells (HUVEC) cells showed a similar attachment. Furthermore, co-culture studies with HUVEC showed that HUVEC protected CLL cells from spontaneous apoptosis by direct cell-to-cell contact as assessed by flow cytometry using Annexin V. Our data suggest that constitutively activated STAT3 induces VEGF production by CLL cells and CLL cells derive a survival advantage from endothelial cells via cell-to cell contact.

Keywords

CLL; STAT3; VEGF; Endothelial cells; Stroma

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Correspondence: Zeev Estrov, M.D. zestrov@mdanderson.org; Address: Department of Leukemia, Unit 402, M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030; Phone: 713-794-1675.

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1. Introduction

Chronic lymphocytic leukemia (CLL) is a B-cell lymphoproliferative disorder characterized by an accumulation of clonal B-lymphocytes in the peripheral blood, bone marrow and lymphatic tissues. The clinical heterogeneity of CLL is influenced by its numerous pathobiological features including intrinsic genetic influences on cell proliferation and survival and extrinsic influences stemming from antigen stimulation, immune regulation and tumor microenvironment. The tumor microenvironment in CLL confers cell survival benefits and resistance to apoptosis. A number of cells have been shown to provide a protective environment to CLL cells including mesenchymal stroma cells, “nurse-like” cells and follicular dendritic cells (1). The microenvironment cellular elements and molecular mechanisms responsible for providing CLL cells with survival advantage have not yet been fully elucidated.

Like in solid tumor tissues and hematologic neoplasm bone marrow (BM), increased microvascular density (MVD) was found in both BM and lymph nodes of patients with CLL (2–5). In-vivo angiogenic assays in the chick embryo chorioallantoic membrane (CAM) have demonstrated that CLL cells induce new vessel formation by producing vascular endothelial growth factor (VEGF) (2). Because VEGF is a potent inducer of angiogenesis, it was postulated that increased MVD in CLL BM is a result of CLL cell-derived VEGF. Notably, CLL patients’ VEGF serum levels are higher than those of healthy individuals and correlate negatively with the patients’ prognosis (6–10). CLL cells also express VEGF receptors (R) including VEGFR1, VEGFR2 and VEGFR3, thereby implying a possible VEGF-mediated autocrine survival mechanism of CLL cells (8, 11–13). Indeed, high VEGFR2 on CLL cells are associated with poor survival (13).

Members of the signal transducers and activators of transcription (STAT) family play a pivotal role in cell survival and proliferation (14). Under physiologic conditions, binding of growth factors or cytokines to their corresponding receptors induces phosphorylation (p) and dimerization of STAT molecules. STAT dimers translocate to the nucleus, bind to DNA and activate transcription (15). In different solid and liquid tumors STAT3 was found to be constitutively phosphorylated on tyrosine residues (16, 17). Unlike in other hematologic malignancies in CLL, STAT3 is constitutively phosphorylated on serine 727, but not tyrosine, residues and activates genes known to be regulated by tyrosine pSTAT3 (14, 18, 19). VEGF is a target gene of phosphotyrosine STAT3 and its production is activated by tyrosine pSTAT3 in several neoplasms (20–22). We hereby report that in CLL phosphoserine STAT3 activates VEGF and affects the interaction between CLL cells and their BM microenvironment.

2. Materials and Methods

2.1. Blood and marrow samples

Peripheral blood (PB) cells and stored bone marrow (BM) biopsy specimens were obtained from patients with CLL who were treated at The University of Texas M. D. Anderson Cancer Center Leukemia Clinic during the years 2006–2008, after obtaining Institutional

Review Board (IRB)-approved informed consent. Samples from untreated or patients that were not treated for the last 6 months were chosen. To isolate low-density cells, PB cells were fractionated using Ficoll Hypaque 1077 (Sigma-Aldrich, St. Louis, MO). More than 95% of the low-density PB lymphocytes obtained from these patients were CD19⁺/CD5⁺. Fractionated CLL cells were maintained in DMEM (Sigma-Aldrich) supplemented with 10% FBS (Hyclone, Logan, UT).

2.2. Cell lines

The human umbilical vein vascular endothelial cells (HUVEC) were obtained from Lonza (Walkersville, MD) and grown in the presence of Lonza's proprietary culture medium. The human mesenchymal (HS-5) cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 (Sigma-Aldrich) supplemented with 10% FBS (Hyclone). The cells were harvested at the peak of their growth and used in the experiments described below.

2.3. Chromatin Immunoprecipitation (ChIP)

The enzymatic ChIP Kit (Cell Signaling Technology) was used. Fractionated CLL cells were cross-linked with 1% formaldehyde for 10 minutes at room temperature, a glycine solution was added to arrest the reaction, and the cells were washed with ice-cold PBS. The cells were pooled, pelleted, and incubated on ice for 10 minutes in cell lysis buffer supplemented with 100 mM phenylmethylsulfonyl fluoride, dithiothreitol (DTT) and a protease cocktail inhibitor mix. Nuclei were pelleted and resuspended in buffer supplemented with DTT, digested by micrococcal nuclease, and homogenized on ice. Following sonication (35 pulses, 20 sec/pulse at 25–30% power) and centrifugation, 12 mg of sheared chromatin was incubated with anti-STAT3 or Rabbit serum (negative control) overnight at 4°C. Then, magnetic-coupled protein G beads were added and the chromatin was incubated for 2 hours in rotation. Antibody-bound protein/DNA complexes were washed, eluted, treated with proteinase digest proteins, and subjected to polymerase chain reaction (PCR) analyses. An aliquot of chromatin that was not incubated with an antibody was used as the input control sample. The primers to amplify the human STAT3 promoter were F: 5-CCG AAC GAG CTG GCC TTT CAT-3 and R: 5-GGA TTG GCT GAA GGG GCT GTA-3 which generated a 86 bp product; to amplify the Waf1/p21 promoter: F: 5-TTG TGC CAC TGC TGA CTT TGT C-3 and R: 5- CCT CAC ATC CTC CTT CTT CAG GCT-3, which generated a 303 bp product; to amplify the c-Myc promoter: F: 5-TGA GTA TAA AAG CCG GTT TTC-3 and R: 5-AGT AAT TCC AGC GAG AGG CAG-3, which generated a 63 bp product; and to amplify the VEGF promoter: R: 5-1453 CCTGGAAATAGCCAGGTCAG F: 3-1272 CTTCTCCAGGCTCACAGCTT. The human RPL30 gene primers were provided by Cell Signaling Technologies. PCR products were resolved on 1.8% agarose gels containing anthodium bromide.

2.4. Generation of green fluorescence protein (GFP)-lentivirus STAT3 shRNA and infection of CLL cells

293T cells were co-transfected with GFP-lentivirus STAT3 small hairpin RNA (shRNA) or GFP-lentivirus empty vector and the packaging vectors pCMVdeltaR8.2 and pMDG (generously provided by Dr. G. Inghirami, Torino, Italy) using the superfect transfection

reagent (Qiagen, Inc.). 293T cell culture medium was changed after 16 hours and collected after 48 hours. The culture medium was filtered through a 45- μ m syringe filter to remove floating cells, the lentivirus was then concentrated by filtration through an Amicon ultra centrifugal filter device (Millipore, Billerica, MA), and the concentrated supernatant was used to infect CLL cells. CLL cells (5×10^6 /mL) were incubated in 6-well plates (Becton Dickinson, Franklin Lakes, NJ) in 2 ml DMEM supplemented with 10% FCS and transfected with 100 μ L of viral supernatant. Polybrene (10 ng/mL) was added to the viral supernatant at a ratio of 1:1000 (v/v). Infection efficiency was measured after 48–72 hours and was found to range between 40% and 70% (calculated on the basis of the ratio of propidium iodide (PI)-negative/GFP-positive cells). These experiments were conducted by using the FACSCalibur flow cytometer and data analysis was performed using its software programs (Becton Dickinson Immunocytometry Systems, San Jose, CA). A shift from the control curve was calculated as percent shift beyond control.

2.5. RNA purification and quantitative real-time (qRT)-PCR

RNA was isolated using an RNeasy purification procedure (Qiagen, Inc., Valencia, CA). RNA quality and concentration were analyzed with a NanoDrop spectrophotometer (ND-1000; NanoDrop Technologies, Wilmington, Delaware). Five hundred nanograms of total RNA was used in one-step qRT-PCR (Applied Biosystems, Foster City, CA), with the sequence detection system ABI Prism 7700 (Applied Biosystems) using TaqMan gene expression assay for STAT3, VEGF, Bcl2, Bcl-X_L, c-Myc, Waf1/p21, Cyclin D1, and S18 according to the manufacturer's instructions. Samples were run in triplicate, and relative quantification was performed by using the comparative C_T method (19).

2.6. CLL-mesenchymal and -endothelial cell co-culture conditions

Fractionated patient CLL cells were incubated for 96 hours with or without either 10% phytohemagglutinin T-cell conditioned medium (PHA-TCM) or 10 ng/ml VEGF (BioSource International). In co-culture experiments CLL cells were incubated with HUVEC or HS-5 cells under conditions that allow direct contact or separated by inserts of micropore filters (Falcon Labware, Becton Dickinson, Franklin Lakes, NJ). In some experiments 20 mcg/ml of anti-VEGFR2 antibodies (Abcam Inc., Cambridge, MA) or 100 mcg/ml AMD3100 (Plerixafor, Sigma) were added.

2.7. Apoptosis assay

The rate of cellular apoptosis was analyzed using double staining with a Cy5-conjugated annexin V kit and propidium iodide (PI) (BD Bioscience, Palo Alto, CA), according to the manufacturer's instructions. Briefly, 1×10^6 cells were washed once with PBS and re-suspended in 200 μ L binding buffer with 0.5 μ g/mL annexin V-Cy5 and 2 μ g/mL PI. After incubation for 15 minutes in the dark at room temperature, the samples were analyzed on a FACSCalibur flow cytometer (Becton Dickinson). Cell viability was calculated as the percentage of annexin V-positive cells and statistical analysis was performed using the CellQuest software.

2.8. Quantitation of plasma VEGF levels

Plasma VEGF levels of 47 patients with untreated CLL and 14 normal donors were measured using a quantitative sandwich enzyme linked immunoassay (Quantikine; R&D Systems, Minneapolis, Minn.) according to the manufacturer's instructions. Briefly, 100 μ l of plasma was added to the VEGF microplate wells and after incubation and washes a polyclonal antibody to VEGF conjugated to horseradish peroxidase was added to the wells. Color was developed by adding tetramethylbenzene as chromogen. Appropriate standards were incorporated.

2.9. Immunohistochemistry

Immunohistochemical staining was performed using 5-micron thick, formalin-fixed, paraffin-embedded tissue sections, epitope retrieval with Diva decloacker buffer and a decloacking chamber (Biocare Medical, Concord, CA), and the Mach 3 system (Biocare Medical). Staining was performed using the Autostainer Plus (DakoCytomation, Carpinteria, CA). The washing buffer used was 0.05-M Tris-buffered saline supplemented with 0.05% Tween. Diaminobenzidine tetrahydrochloride was used as the chromogen, and all tissue sections were counterstained with hematoxylin. Single antibody staining was conducted using specific antibodies for VEGF (1:100; Rabbit anti-VEGF; Invitrogen Corporation, Carlsbad, CA), VEGFR2 (Flk-1 or A-3; 1:200, mouse anti-human VEGFR2; DakoCytomation) and CD34 (1:200, Biocare Medical). Specimens were considered positive if 20% or more of the CLL cells stained positively. Both membrane and cytoplasmic staining were recorded separately.

The degree of vascular density was quantified using the Chalkley counting procedure as proposed in the International Consensus Meeting (23). The degree of angiogenesis was quantified in bone marrow trephine biopsy sections that had a nodular, interstitial, or diffuse involvement with untreated chronic lymphocytic leukemia (n= 9), comparing it to normal control bone marrow sections (n=9). The blood vessels in bone marrow sections were highlighted by CD34 immunohistochemical staining. We used the Chalkley point overlap morphometric technique (23). Sections stained with anti-CD34 antibodies were scanned at low power (at X40 and X100 magnifications), and then three areas, considered to have the highest vascularity in the section (hotspots), were selected. Then, a 25 point Chalkley eyepiece graticule was applied to each hotspot area at X200 magnification with a Chalkley grid area of 0.1886 mm² (Olympus microscope BX41). The dots in the graticule hitting any stained vessel or endothelial cell were counted in each hotspot selected. The highest score of the three hotspot counts was used for analysis. To correct for cellularity, we multiplied the Chalkley counts in the control samples by a factor equal to the difference in cellularity between CLL and control bone marrows. Double antibody staining was conducted by using first VEGF (1:100, RT for 60 minutes; Dako, Carpinteria, CA) and Fast-Red substrate (DakoCytomation) as the chromogen. The tissue sections were rewashed in buffer, double-stain block (DakoCytomation) was applied for 3 minutes, and the sections were incubated with the second anti-VEGFR2 antibody, as described above. After the reaction was visualized with diaminobenzidine tetrahydrochloride the tissue sections were then counterstained with hematoxylin.

2.10. Statistical analysis

Student's *t*-test was used to determine the significance of differences between different variables.

3. Results

3.1. Stat3 binds to the VEGF gene promoter in CLL cells

We have recently demonstrated that STAT3, constitutively phosphorylated on serine 727 residues in CLL cells, activates genes known to be activated by phosphotyrosine Stat3 (19). Because Stat3 modulates the expression of VEGF gene in other cellular systems (20–22, 24), we asked whether Stat3 activates the VEGF gene in CLL cells. We first obtained fresh low-density CLL PB cells and performed a ChIP assay. As shown in Fig. 1A, anti-Stat3 antibodies coimmunoprecipitated DNA of VEGF, Stat3, and the stat3-regulated genes Waf1 and C-Myc from CLL cell chromatin implying that similar to other Stat3-regulated genes, Stat3 binds to VEGF promoter in CLL cells.

3.2. STAT3 shRNA downregulates VEGF RNA levels in CLL cells

To determine whether serine pSTAT3 regulates VEGF gene expression we infected CLL cells with STAT3-shRNA and quantitated VEGF and Stat3-regulated gene RNA levels using qRT-PCR. As shown in Fig. 1B, STAT3-shRNA significantly downregulated RNA levels of VEGF and the Stat3-regulated genes STAT3, Bcl-2, Bcl-X_L, cyclin D1, c-Myc and Waf1 by 6 to 12 fold when compared to control lentivirus empty vector. Taken together, these data suggest that Stat3 binds to the VEGF promoter and induces VEGF gene expression.

3.3. CLL cells express and secrete high levels of VEGF protein

Next we sought to determine whether CLL cells produce VEGF. We obtained BM biopsy samples from 9 patients with CLL and using immunochemistry detected intracellular VEGF protein. When compared with normal marrow, CLL marrow lymphocytes expressed high levels of VEGF. Similarly, BM CLL cells expressed high levels of VEGFR2 (Fig. 2, upper panel), as previously reported (13). Then, to determine whether VEGF could be detected in CLL patients' serum we performed ELISA using serum samples for 47 patients with CLL and 14 hematologically normal individuals. VEGF levels were significantly higher in CLL than in normal plasma and VEGF levels correlated with the patients' white blood cell count (Fig. 2, lower panel).

3.4. CLL cells segregate in the vicinity of vascular endothelial cells

Because VEGF induces microvessel formation (25) we wondered whether CLL BM encompasses increased vasculature. Consistent with previous reports (3–5) we found increased microvasculature in 9 out of 9 CLL bone marrow biopsies as compared with bone marrow biopsies that were obtained from 9 hematologically normal individuals (Fig. 3). In addition, we found that CLL cells segregate around vascular endothelial cells (Fig. 3D).

3.5. CLL cells attach to HUVEC

To explore the interaction between CLL and vascular endothelial cells, we obtained HUVEC and grew them in plastic flasks under their optimal culture conditions. After the cells formed a thin non-confluent adherent layer we added fresh CLL cells to the culture. As shown in Fig. 4A, within a few hours CLL cells homed to and attached to the adherent HUVEC cells.

3.6. HUVEC protect CLL cells from spontaneous apoptosis through direct cell to cell contact

To explore how vascular endothelial cells affect CLL cells, we performed co-culture experiments of patient-derived CLL cells and HUVECs. As control we used human mesenchymal (HS-5) cells. CLL cells were cultured for 96 hours in RPMI supplemented with PHT-TCM, VEGF or co-cultured with HUVEC or HS-5 cells with or without inserts. After 96 hours CLL cells were removed from culture and cellular apoptosis was assessed using Annexin V. As shown in Fig. 4B after 96 hour incubation with PHA-TCM CLL cell apoptosis increased from 5.2% to 37.8%. Similarly, 31.9% and 29.4% of the cells in co-culture with HS-5 cells were apoptotic whether incubated without or with an insert, respectively. Co-culture with HUVEC reduced CLL cell apoptosis to 24.7% and the protective effect was diminished when CLL cells were placed in an insert that did not allow direct cell-to-cell contact. Phagocytosis was not observed in any of our culture.

3.7. VEGF protects CLL cells from apoptosis directly not through VEGF-VEGFR2 interaction

Because we found that HUVEC protect CLL cells from apoptosis by a direct cell-to-cell interaction and, as shown in Fig. 2, endothelial cells express VEGFR2 we wondered whether an interaction of CLL cells with VEGFR2 plays a role in protecting CLL cells from apoptosis. As shown in Figs. 4C and 5D, VEGFR2 neutralizing antibodies did not diminish the protective effect exerted by HUVEC under culture conditions that allow cell-to-cell contact. In contrast, VEGF by itself significantly protected CLL cells from apoptosis and VEGF neutralizing antibodies suppressed this effect (data not shown). Several investigators demonstrated that CLL cells express CXCR4 and that CXCL12-expressing BM stroma cells protect CLL cells from apoptosis via CXCR4-CXCL12 interaction (1). Similar to BM vascular endothelial cells, HUVEC express CXCL12 (26). To target the CXCR4-CXCL12 interaction, the CXCR4 chemokine receptor antagonist Plerixafor (27) was added to HUVEC-CLL cell co-culture. As shown in Fig. 4E, Plerixafor did not directly affect the survival of CLL cells but rather increased the spontaneous apoptosis rate of CLL cells co-cultured with HUVEC by 7.9%, suggesting that CXCR4-CXCL12 interaction plays a role in the protective effect exerted by HUVEC.

4. Discussion

In the current study we analyzed bone marrow biopsies of nine patients with CLL and, using immunohistochemistry, we confirmed previous studies showing that CLL cells express cell surface VEGFR2 and produce high quantities of VEGF (2, 6–8, 10, 11, 13, 28–31). In addition, as reported by other investigators (3–5, 31), we found increased MVD in all BM

specimens. CLL patients' plasma VEGF levels were higher than healthy individuals' levels and correlated with WBC count, confirming previously published data (2, 6–8, 10).

The mechanism by which VEGF is upregulated in CLL cells has been recently demonstrated to be dependent on the Von-Hippel Lindau (pVHL) gene product. Ghosh et al. demonstrated that Hypoxia inducible factor (HIF)-1 α was constitutively upregulated in CLL cells in normoxic conditions, as a consequence of suppression of pVHL by micro RNA (miR) 92-1. HIF-1 α formed a nuclear complex with the p300 transcriptional factor and serine pSTAT3 and this complex bound the VEGF DNA promoter. However, STAT3 is constitutively activated in CLL (4). STAT3 is required for both basal and signal-induced expression of HIF-1 α (22) and inhibits the degradation of HIF-1 α by pVHL-mediated ubiquitination (32, 33). Thus, STAT3 is probably responsible for constitutive upregulation of HIF-1 α in CLL cells. Furthermore, pSTAT3 has been shown to induce VEGF production (20–22) and high VEGF levels correlate with WBC count and unfavorable prognosis in CLL (6, 9, 10, 13). Therefore we postulated that STAT3 induces VEGF production in CLL.

Previous studies demonstrated that tyrosine pSTAT3 binds to the VEGF gene promoter and induces VEGF protein production (20–22). However, tyrosine pSTAT3 has not been detected in unstimulated CLL cells. Rather, STAT3 is constitutively phosphorylated on serine, but not tyrosine, residues (7, 11, 13, 31). Because we recently found that in CLL cells serine pSTAT3 is biologically active and activates genes known to be modulated by phosphotyrosine STAT3 (19), we wondered whether serine pSTAT3 induces VEGF. Using ChIP we found that anti-STAT3 antibodies co-immunoprecipitated DNA of STAT3, VEGF, and the STAT3-regulated genes p21 and c-Myc, and observed downregulation of RNA levels of VEGF and the STAT3-regulated genes Bcl-2, Bcl-X_L, cyclin D1, c-Myc, and Waf1 following transfection of CLL cells with STAT3-shRNA. Taken together, these data suggest that serine pSTAT3 induces the production of VEGF in CLL cells.

Analysis of CLL BM biopsies revealed that vascular endothelial cells express cell surface VEGFR2 and that CLL cells segregate around vascular endothelial cells. Because the interaction between CLL cells and their BM microenvironment plays an important role in the pathobiology of this disease, we postulated that the endothelial component of CLL BM stroma provides CLL cells with survival advantage. To delineate this interaction we used HUVEC. Using co-culture experiments we found that HUVEC protected CLL cells from undergoing spontaneous apoptotic cell death. However unlike the effect of the mesenchymal HS-5 cells, HUVEC's protective effect was contact-dependent. The ability of vascular endothelial cells to confer protection to CLL cells has been previously described by Long et al. (34). Similar to us, they found that the protective effect was not mediated by a soluble factor but by direct cell-to-cell contact. Thus, VEGF produced by CLL cells stimulates vascular endothelial cell proliferation resulting in angiogenesis and increased MVD, and the endothelial cells, in turn, provide CLL cells with survival advantage.

That VEGF binds to endothelial cell VEGFR2 and activates signal transduction has been previously reported and the VEGF-VEGFR2 interaction has been thoroughly studied (see reviews (35, 36)). However, we found that the anti-apoptotic effect exerted by endothelial (HUVEC) cells is not mediated via a VEGF-VEGFR2 interaction. In co-culture studies

VEGFR2 neutralizing antibodies did not diminish the protective effect. We then asked whether the anti-apoptotic effect exerted by the vascular endothelial cells depends on the interaction between CXCR4 and CXCL12. CLL cells expressing CXCR4 interact with CXCL12-expressing marrow stromal cells (reviewed in (1)) and HUVEC express CXCL12 (26). We added the CXCR4 inhibitor Plerixafor to our co-culture system and found that Plerixafor partially reversed the anti-apoptotic effect exerted by HUVEC. It is possible that another integrin-mediated mechanism is engaged, as suggested by Long et al. This assumption is supported by a recent study showing that unlike normal B cells, CLL lymph node cell migration and mobilization through the vascular endothelium is dependent on binding to the $\alpha 4 \beta 1$ integrin and that this interaction is dependent on an autocrine VEGF-VEGFR interaction (18).

In agreement with previous reports (28, 37, 38), we found that excess VEGF protected CLL cells from spontaneous apoptosis. This effect might require activation of additional mechanisms. Farahani et al. Found that VEGF exerts an antiapoptotic effect on CLL cells only when CD40 is costimulated (37). The expression of the CD40 ligand CD154 is increased following exposure to interleukin-4 (39), whose levels are increased in CLL (40).

STAT3 is constitutively phosphorylated on serine 727 residues in all patients with CLL regardless of clinical characteristics, disease stage, or treatment status (18, 19). Serine pSTAT3 translocates to the nucleus, binds to DNA, and activates the transcription of STAT3-regulated genes known to affect cell proliferation and survival (38). Our data suggest that constitutive phosphoserine STAT3 activates VEGF thereby affecting the cross-talk between CLL cells and their BM microenvironment.

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Fig. 1A

Fig. 1B

Figure 1. STAT3 upregulates VEGF gene expression

A. STAT3 co-immunoprecipitates with DNA of VEGF and STAT3-regulated genes. CLL cell-derived chromatin was immunoprecipitated by anti-STAT3 antibodies or rabbit serum (IgG control). The co-immunoprecipitated DNA was amplified by PCR using promoter constructs of STAT3, VEGF, waf1/p21, c-Myc, or the control gene RPL30. As shown, DNA of STAT3, VEGF, waf1/p21, and c-Myc, but not the control RPL30 gene, co-immunoprecipitated with STAT3 protein. STAT3, waf1/p21, and c-Myc, and RPL30 DNA was detected in whole cell chromatin-extracted DNA (Input). The data depicted were generated using CLL cells of a randomly chosen patient. Similar data were generated using cells from two other patients. **B.** STAT3-shRNA downregulates mRNA levels of STAT3, VEGF and STAT3-regulated genes. CLL cells were infected with a lentiviral STAT3-shRNA and, as control, with the empty vector. CLL cell mRNA levels were assessed using qRT-PCR. As shown, STAT3-shRNA downregulated mRNA levels of STAT3, VEGF, Bcl2, Pim1, Bcl-X_L, p21 (Waf1), and c-Myc. Changes in mRNA levels (mean $\pm$ SD) are shown. The qRT-PCR results are depicted as fold change (decrease or increase) relative to the change in mRNA levels of CLL cells infected with the lentiviral empty vector (control).

Figure 2. CLL cell express levels of VEGF and VEGFR2 and VEGF plasma levels correlate with WBC count

Upper panel: BM biopsy specimens were stained with hematoxylin-eosin (HE) and by immunohistochemistry using anti-VEGF and anti-VEGFR2 antibodies. High VEGF and VEGFR2 levels are detected in CLL cells when compared to BM lymphocytes of hematologically normal individuals. Lower panel: Plasma VEGF levels from 47 patients with CLL and 14 healthy individuals were assessed using ELISA. As shown, plasma VEGF levels were significantly higher in patients with CLL than in normal individuals and VEGF levels correlated with peripheral blood WBC count.

Figure 3. CLL bone marrow is characterized by increased microvascular density and CLL cells express VEGF and VEGFR2

Left panel: Microphotograph of bone marrow trephine biopsy sections stained for CD34 and measured using Chalkley method. Bone marrow sections have more microvessels as compared to normal sections. **A.** There are numerous microvessels in a CLL bone marrow core section, highlighted by CD34 immunohistochemical staining (brown). **B.** In comparison, normal control bone marrow sections had fewer microvessels. Two microvessels (brown) are evident in this field. All images were taken by a SPOT Insight color digital camera at $\times 20$ magnification with a Plan 20x/0.40 objective lens in Olympus BX41 microscope. Right panel: CLL BM biopsies from 9 patients were immuno-stained with both VEGF and VEGFR2. Representative fields are depicted. **C.** Both CLL cells and vascular endothelial cells express VEGFR2 (brown). A microvessel (arrows) and CLL cells containing VEGF (red) are shown. **D.** CLL cells attach to vascular endothelial cells expressing VEGFR2 (arrows).

Fig. 4A

Fig. 4B

Fig. 4C

Fig. 4D

Fig. 4E

Figure 4. Interaction between CLL cells and endothelial cells

A. A photomicrograph (X 40) of CLL cells added to an established culture of adherent endothelial cells (HUVEC) depicts homing of CLL cells to plastic-adherent HUVEC. Elongated cells are HUVECs, round cells are CLL cells and the dark dots are out of focus CLL cells and cell debris. **B.** Apoptosis analysis by Annexin V and propidium iodide (PI) using flow cytometry of CLL cells harvested from co-culture experiments. CLL cells were incubated for 96 hours in growth media. The percentage of apoptotic CLL cells were calculated by quantitating Annexin V positive cells as assessed by flow cytometry; the percentages of early apoptotic cells are shown in the bottom right corners and of late apoptotic (PI+ and annexin V+) cells in the top right corners. Results of apoptosis analysis demonstrate a significant decrease in apoptosis when CLL cells were co-cultured with HUVECs or with addition of VEGF when compared to control medium. In contrast, co-culture with mesenchymal (HS-5) cells a lesser protective effect was observed. Use of a micropore insert to prevent direct contact between HUVECs and CLL cells abrogated the protective effect of vascular endothelial cells but did not change the protective effect resulting from co-culture with HS-5 cells. Representative data from four different experiments are depicted. **C.** Co-culture experiments performed with CLL cells in the presence or absence of vascular endothelial cells. CLL cells exposed to VEGF or co-cultured with HUVEC had significantly lower rates of apoptosis compared to control cells. Insertion of a micropore membrane reversed the protective effect of HUVEC. Anti-VEGFR2 neutralizing antibodies did not reverse the protective effect of HUVECs on CLL cells, suggesting that signaling through VEGFR2 is not required for this protective effect. Representative data from four different experiments are depicted. VEGF alone (in the absence of HUVEC) was added and its effect was compared to control. **D.** Summary of data from 4 co-culture experiments of CLL cells with HUVEC, with or without insertion of

micropore membrane or addition of anti-VEGFR2 antibodies or VEGF. Percentages (mean $\pm$ SD) of AnnexinV⁺/PI^{+/-} CLL cells are depicted. **E.** Effect of the CXCR4 inhibitor Plerixafor on the interaction between HUVEC and CLL cells. As in our previous experiments co-culture with HUVEC reduced the spontaneous apoptosis rate of CLL cells at from 44.7% (left upper corner) to 28.8% (right upper corner). Assessment of AnnexinV⁺/PI^{+/-} CLL cells was conducted at 48 hours after initiation of culture. Plerixafor (100 mg/ml) did not affect the apoptosis rate of CLL cell in liquid culture (left upper Vs left lower corner) however it increased the apoptosis rate of CLL cells co-cultured with HUVEC by 7.9% (right upper corner Vs. right lower corner).