Matrix metalloproteinase-9 induces a pro-angiogenic profile in chronic lymphocytic leukemia cells

Noemí Aguilera-Montilla[a], Elvira Bailón[a], Estefanía Ugarte-Berzal[b], Rebeca Uceda-Castro[a], María Prieto-Solano[a], Elena García-Martínez[c], Rafael Samaniego[c], Philippe E. Van den Steen[b], Ghislain Opdenakker[b], José A. García-Marco[d], and Angeles García-Pardo[a*]

[a]Department of Molecular Biomedicine, Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas (CSIC), Madrid, Spain; [b]Rega Institute for Medical Research, Department of Microbiology, Immunology and Transplantation, KU Leuven, Belgium. [c]Immuno-oncology Laboratory, Confocal Microscopy Unit, Instituto de Investigación Sanitaria Gregorio Marañón, Madrid, Spain. [d]Hematology Department, Hospital Universitario Puerta de Hierro, Madrid, Spain.

Correspondence: Dr. Angeles García-Pardo, Department of Molecular Biomedicine, Centro de Investigaciones Biológicas, CSIC. Ramiro de Maeztu 9, 28040 Madrid, Spain. Tel: 34 91 8373112 ext 4430; E-mail: agarciapardo@cib.csic.es

ABSTRACT

Increased angiogenesis is commonly observed in chronic lymphocytic leukemia (CLL) tissues in correlation with advanced disease. CLL cells express pro- and anti-angiogenic genes and acquire a pro-angiogenic pattern upon interaction with the microenvironment. Because MMP-9 (a microenvironment component) plays important roles in solid tumor angiogenesis, we have studied whether MMP-9 influenced the angiogenic pattern in CLL cells. Immunofluorescence analyses confirmed the presence of MMP-9 in CLL tissues. MMP-9 interaction with CLL cells increased their MMP-9 expression and secretion into the medium. Accordingly, the conditioned media of MMP-9-primed CLL cells significantly enhanced endothelial cell proliferation, compared to control cells. MMP-9 also increased VEGF and decreased TSP-1 and Ang-2 expression, all at the gene and protein level, inducing a pro-angiogenic pattern in CLL cells. Mechanistic analyses demonstrated that downregulation of the selected gene *TSP-1* by MMP-9 involved $\alpha 4\beta 1$ integrin, Src kinase family activity and the STAT3 transcription factor. Regulation of angiogenic genes is a novel contribution of MMP-9 to CLL pathology.

Keywords: CLL, MMP-9, angiogenic gene regulation, thrombospondin-1

1. Introduction

Chronic lymphocytic leukemia (CLL), the most common leukemia in Western countries, is characterized by the accumulation of malignant B-lymphocytes in peripheral blood and lymphoid organs [1,2]. The microenvironment in these

niches provides proliferative and survival signals for CLL cells, contributing to progression of the disease [3].

Angiogenesis, the development of new microvessels from preexisting ones, is another active process in CLL organs and microvessel density correlates with advanced clinical stages [4,5]. CLL cells constitutively express and release pro- and anti-angiogenic molecules, including fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), thrombospondin-1 (TSP-1), and angiopoietin-2 (Ang-2) [6,7]. Accordingly, the conditioned medium (CM) of CLL cells increases tube formation in *in vitro* assays [8] and elevated serum/plasma levels of some of these factors correlate with advanced CLL [9]. Hypoxic conditions and/or stromal components in CLL tissues regulate the expression of pro/anti-angiogenic factors and induce a pro-angiogenic phenotype in CLL cells [10-12]. Angiogenesis in CLL organs is therefore influenced by the crosstalk between CLL cells and their microenvironment.

Matrix metalloproteinase-9 (MMP-9) is another pro-angiogenic molecule produced by CLL cells [13-17]. A coordinated regulation of MMP-9 and VEGF has been observed in several tumors, suggesting functional links between both molecules [15]. Thus, VEGF enhanced MMP-9 expression and metastasis in ovarian and lung cancer cells [15]. Likewise, MMP-9 was shown to increase the secretion of VEGF and other angiogenic factors in several tumors by releasing these from the extracellular matrix [13-15].

In CLL, we previously demonstrated that VEGF transcriptionally downregulated MMP-9 and inhibited cell migration [18]. It is not known whether MMP-9 regulates angiogenic molecules in CLL. We recently showed

that MMP-9 affects the expression of genes involved in cell adhesion and migration of CLL cells [19]. In the present report we have studied whether MMP-9, a microenvironment component, regulated the expression of pro- and anti-angiogenic factors in CLL. We show that MMP-9 binding to CLL cells upregulates VEGF and MMP-9 and downregulates TSP-1 and Ang-2 at the transcriptional and protein level, inducing a pro-angiogenic profile. We have further established the signaling pathway by which MMP-9 downregulates the selected gene *TSP-1*.

2. Materials and Methods

2.1. Patients and cells

Approval was obtained from the CSIC Bioethics Review Board for these studies. Peripheral blood samples from 30 CLL patients (Table 1) were obtained after informed consent and B-lymphocytes were purified as described [17-19]. Human umbilical vein endothelial cells (HUVEC) were purchased from Lonza (Basel, Switzerland) and cultured in EGM™ Endothelial Cell Growth Medium BulletKit™ (Lonza).

2.2. Immunofluorescence analyses

Paraffin-embedded slides corresponding to four CLL lymph nodes (LN), two CLL bone marrow (BM), and two non-CLL LN samples were de-paraffinized, rehydrated and unmasked by steaming in 10 mM sodium citrate buffer (pH 6.0) for 20 min. Slides were blocked for 30 min with 5 μg/ml human IgG in blocking serum-free medium (Dako) and incubated with 5 μg/ml primary Abs (4°C, overnight). Slides were washed with PBS-Tween 0.05% and incubated

with Alexa Fluor 488- or Alexa Fluor 568-secondary Abs (RT, 1 h). After washing, samples were mounted with fluorescence mounting medium (Dako) and imaged with a Leica SPE confocal microscope using the glycerol-immersion ACS APO 20x/NA 0.60 objective.

2.3. Quantitative PCR (qPCR)

Total RNA from 5 x 10^6 cells was isolated using the Allprep® DNA/RNA/Protein kit (Qiagen, Hilden, Germany) and reverse-transcribed using Moloney murine leukemia virus RT (Fermentas GmbH, St. Leon-Rot, Germany). qPCR was performed using iQTM SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA) and the oligonucleotides listed in Table 2. All assays were performed in triplicate and the results were normalized according to the expression levels of TBP (TATA-binding protein) and expressed using the ΔCT method for quantification.

2.4. RNA interference experiments

siRNA sequences targeting the various human genes studied and control siRNA (Table 2) were custom-made by Sigma-Aldrich. 15 x 10^6 CLL cells were nucleofected with 6 μM siRNAs using Human B cell solution and program U-15 (Amaxa, Cologne, Germany). After 24 h, cells were plated on MMP-9 or BSA-coated plates and cultured for an additional 24 h period.

2.5. Macrophage differentiation

Peripheral blood monocytes from healthy donors were purified from buffy coats using CD14 microbeads (Miltenyi Biotech, Bergisch Gladbach,

Germany) and cultured for 7 days at 0.5 x 10^6 cells/ml in RPMI 1640 (Lonza), 10% FBS, and in the presence or absence of conditioned medium (CM, 1/3 v/v) from CLL cells cultured in RPMI, 10% FBS for 24 h. GM-CSF (1000 U/ml) or M-CSF (10 ng/ml) (Immunotools GmbH, Friesoythe, Germany), were added every 2 days to generate GM-CSF–polarized (GM-MØ) or M-MØ, respectively. Cells were collected after 48 h and 7 d for qPCR analysis.

2.6. HUVEC proliferation assays

3 x 10^4 HUVEC were cultured for 24 h in the presence of conditioned medium from 10 x 10^6 CLL cells that had been incubated for 24 h on MMP-9 (110 nM) or 0.5% BSA coated plates, in RPMI, 0.05% FBS medium. 50 µg of MTT reagent (Sigma Aldrich) was added to the culture 4 h before the end of the assay. The blue MTT formazan precipitate was dissolved in isopropanol-HCl (24:1) and the absorbance at 540 nm was determined on a Multiskan Bichromatic microplate reader (Labsystems, Helsinki, Finland).

Additional methods are available as Supplemental Material.

3. Results

3.1. Exposure of CLL cells to MMP-9, a component of CLL tissues, increases CLL cell effect on endothelial cell proliferation

Increased angiogenesis is a common feature in CLL and is likely influenced by CLL cell interactions with microenvironment components, such as macrophages [4,5,20]. To determine whether CLL cells induced a pro-angiogenic profile in macrophages, peripheral blood monocytes were differentiated into GM-MØ (M1-like) or M-MØ (M2-like) phenotypes in the

presence or absence of CM from 24 h CLL cell cultures. MMP-9 expression was selected as pro-angiogenic pattern marker [13]. After 7 days, the CLL-derived CM increased MMP-9 in both MØ types at the gene (Figure 1A) and protein (Figure 1B) level, compared to control cells. In agreement with this, immunofluorescence analyses of four LN and two BM samples from CLL patients demonstrated the presence of MMP-9 in both tissues, partially associated with MØ (CD68+ cells) (representative cases for LN and BM shown in Figure 1C). Little MMP-9 and colocalization with MØ was observed on non-CLL LN (Figure 1C).

We next studied whether MMP-9 interaction with CLL cells affected their capacity to influence angiogenesis. The possible effect on MMP-9 production was first determined by culturing CLL cells from 12 patients on immobilized MMP-9 for 24 h. qPCR analyses showed that MMP-9 binding to CLL cells significantly increased MMP-9 gene expression, compared to control cells (Figure 1D). ELISA assays demonstrated that secretion of MMP-9 to the medium was also significantly increased upon culturing CLL cells on MMP-9, but not on the control BSA (Figure 1E). This increase in secreted MMP-9 was not observed when no cells were added to the plate (Figure 1E).

The effect of MMP-9-primed CLL CM on endothelial cell proliferation was then addressed. HUVEC cells were incubated with CM from CLL cells cultured on MMP-9 or BSA, and HUVEC proliferation was measured after 24 h by the MTT method. Figure 1F shows that the CM from MMP-9-primed cells significantly enhanced HUVEC proliferation, compared to the CM from BSA-treated cells. Altogether these results demonstrated that MMP-9 is actively

produced and present in CLL tissues and that MMP-9 interaction with CLL cells enhances their proliferative effect on endothelial cells.

3.2. MMP-9 regulates the expression of pro- and anti-angiogenic genes in CLL cells

CLL cells were previously shown to acquire a pro-angiogenic profile upon interaction with stromal cells [10-12]. The results shown above indicated that MMP-9, a stromal component in CLL tissues, increased MMP-9 expression in CLL cells, likely via autocrine and paracrine pathways. To determine if other pro/anti-angiogenic genes were also regulated by MMP-9, CLL cells were cultured on BSA or MMP-9 for 24 h and the expression of selected genes was analyzed. qPCR analyses indicated that CLL cell binding to MMP-9 increased the expression of the pro-angiogenic gene *VEGF*, while the anti-angiogenic *TSP-1* and *Ang-2* genes were downregulated (Figure 2A). MMP-9 did not significantly affected *bFGF* expression (Figure 2A). Moreover, Western blotting analyses of lysates of the same CLL cells demonstrated that MMP-9 also significantly upregulated VEGF and downregulated TSP-1 and Ang-2 at the protein level (Figure 2B). These results indicated that isolated MMP-9 was also able to induce a pro-angiogenic pattern in CLL cells.

3.3. TSP-1 downregulation by MMP-9 involves $\alpha 4\beta 1$ integrin, SFK activity and the STAT3 transcription factor

TSP-1 is a well-known angiogenesis inhibitor [21] and was selected for further mechanistic analyses. We previously showed that MMP-9 binding to $\alpha 4\beta 1$ integrin in CLL cells induces several functional effects on these cells

[19,22,23]. To determine whether regulation of *TSP-1* involved binding of MMP-9 to $\alpha4\beta1$, CLL cells were transfected with two different $\alpha4$ siRNAs ($\alpha4_1$, $\alpha4_2$), prior to exposure to MMP-9. qPCR analyses confirmed that both siRNAs significantly reduced $\alpha4$ expression (average 48% and 46% reduction, respectively, for $\alpha4_1$ and $\alpha4_2$), compared to cells transfected with control siRNA (Figure 3A). Gene silencing $\alpha4$ integrin partially prevented *TSP-1* downregulation in response to MMP-9, with a 2-fold significant effect ($\alpha4_1$ siRNA) and a 2.7-fold trend ($\alpha4_2$ siRNA), compared to cells transfected with control siRNA (Figure 3B). These results indicated that $\alpha4$ integrin contributed to the MMP-9 regulatory effect.

To further explore the signaling pathway involved in *TSP-1* regulation, CLL cells were cultured on BSA or MMP-9 in the absence or presence of specific kinase inhibitors. Figure 3C shows that blocking BTK or Akt signaling with ibrutinib and triciribine, respectively, did not prevent the down-regulatory effect of MMP-9 on *TSP-1* expression. However, blocking SFK signaling with saracatinib significantly inhibited this effect in the four samples analyzed (Figure 3C), thus implying the activity of this kinase family in the MMP-9-induced regulation of *TSP-1*.

TSP-1 expression may be regulated by several transcription factors, including STAT3 [24,25]. Because we previously showed that MMP-9 binding to $\alpha4\beta1$ integrin activates STAT3 in CLL cells [23], we studied the effect of STAT3 gene silencing on the regulation of *TSP-1*. CLL cells were transfected with two different STAT3 siRNAs and analyzed by qPCR. Figure 3D shows that both siRNAs efficiently reduced STAT3 expression in the seven samples studied. Exposure of STAT3-silenced cells to MMP-9 for 24 h and subsequent

qPCR analyses revealed that *TSP-1* downregulation was less potent in these cells, rendering 2.2-fold ($STAT3_1$ siRNA) and 1.7-fold ($STAT3_2$ siRNA) higher *TSP-1* values than in control siRNA-transfected cells (Figure 3E). Altogether these results established that $\alpha 4\beta 1$ integrin/SFK/STAT3 is a mechanism by which MMP-9 negatively regulates *TSP-1* expression in CLL cells.

4. Discussion

We previously showed that MMP-9 regulates CLL cell migration, arrest and survival, thus favoring disease progression [17,19,22,23]. To further understand the pathological functions of MMP-9 in CLL, in the current study we have addressed the possible role of MMP-9 in the regulation of angiogenic genes in the leukemic cells. Using primary CLL samples from 30 patients, we demonstrate that MMP-9 induces a pro-angiogenic pattern in CLL cells and increases the proliferative effect of CLL CM on endothelial cells.

Previous studies have shown that co-culturing CLL cells with BM derived stromal cells induces a pro-angiogenic switch that favors leukemic cell survival [10,11,26]. MMP-9 is a well-known pro-angiogenic molecule [13-15] and our current results confirm its presence in CLL tissues. The cells responsible for the production of this MMP-9 include macrophages, as we show here. The CM of CLL cells increased MMP-9 expression in both GM-MØ and M-MØ macrophages, providing a clear example of the cross-talk between leukemic cells and their microenvironment [27]. CLL cells also likely contribute to MMP-9 presence in tissues since they express and secrete MMP-9 [16,17].

CLL cells in tissues are thus exposed to endogenous and exogenous MMP-9. In this report, we have approached this pathophysiological

environment by culturing CLL cells with MMP-9. We demonstrate that MMP-9 upregulated the pro-angiogenic genes VEGF and MMP-9 and downregulated the angiostatic genes Ang-2 and TSP-1, thus inducing an angiogenic profile. This profile likely impacts on the angiogenesis observed in CLL niches [4,5], as our results also show that treatment with MMP-9 enhanced the CLL cell proliferative effect on endothelial cells. The regulation of MMP-9 in cells treated with MMP-9 is interesting and may represent an autocrine positive feedback loop, resembling that described for VEGF in endothelial cells [9]. Our present results are the first to show that MMP-9 is a key contributor to the angiogenic switch previously observed in CLL-stromal cell co-cultures.

Some of the genes/proteins regulated by MMP-9 are well studied and play additional functions in CLL not related to angiogenesis. Thus, MMP-9, VEGF, and Ang-2 were shown to modulate cell survival and migration, having pathological roles in CLL [9, 17,19, 22, 23, 28]. VEGF and Ang-2 were also involved in the angiogenesis induced by CLL cells [8]. Moreover, elevated plasma/serum levels of VEGF and Ang-2 were shown to correlate with advanced CLL, and both molecules were proposed as prognostic markers [7, 9, 29]. Little is known on the function of TSP-1 in CLL, but TSP-1 is a potent anti-angiogenic factor [21], downregulated in stromal cell-CLL cell cocultures [10,11], and it was selected for further mechanistic studies. Using gene silencing and kinase inhibition approaches, we demonstrate that downregulation of *TSP-1* by MMP-9 involves $\alpha 4\beta 1$ integrin, SFK and STAT3 signaling. We previously reported that MMP-9 binding to $\alpha 4\beta 1$ integrin increased CLL cell survival by upregulating Mcl-1 via activation of Lyn kinase and STAT3 [23]. The fact that Lyn is a member of the SFK and a main kinase

expressed by CLL cells [23], strongly suggests that Lyn may be the SFK involved in *TSP-1* downregulation. MMP-9 may therefore utilize the $\alpha 4\beta 1$/SFK/STAT3 pathway to exert various functions in CLL. Interestingly, MT1-MMP (another MMP) regulated VEGF in breast carcinoma cells via the SFK member Src [30], suggesting that SFK activation may be a common mechanism by which MMPs modulate angiogenic genes.

A critical role for STAT3 in the regulation of VEGF and angiogenesis has been previously established [24]. It was also shown that STAT3 enhanced angiogenic factors and suppressed angiostatic molecules (including TSP-1) in cardiomyocytes, favoring angiogenesis [24]. Opposite regulation of *VEGF* and *TSP-1* by STAT3 was also induced by the MMP ADAM12 in breast carcinoma cells [31]. In CLL, STAT3 is constitutively phosphorylated on serine 727, upregulates VEGF and affects the cross-talk between CLL cells and BM endothelial cells [26]. We now show that silencing STAT3 prevented the downregulatory effect of MMP-9 on *TSP-1*, indicating that STAT3 suppresses *TSP-1* expression in CLL cells in response to MMP-9. It is likely that the concomitant upregulation of VEGF observed in our study also involves STAT3, as this factor may play a central role in the various pathological effects induced by MMP-9 in CLL cells.

Although the specific role(s) of TSP-1 in CLL is unknown, TSP-1 plasma levels are elevated at the initial stages of the disease, correlate with good prognostic markers, and decrease as CLL progresses [11]. Contrarily, VEGF plasma levels increase in advanced CLL [7,9]. Our present results on the opposite modulation of these molecules by MMP-9 are therefore relevant

in the pathophysiology of CLL and reveal a novel role of MMP-9 contributing to disease progression.

Authorship

NA-M, EB, and EU-B performed research, designed some experiments, and analyzed data. RU-C and MP-S performed some experiments and analyzed data. EG-M and RS performed and analyzed the immunofluorescence experiments. JAG-M contributed patient samples and clinical data. PEVdS and GO prepared and characterized the recombinant MMP-9 protein and critically reviewed the manuscript. AG-P designed and supervised research and wrote the paper. All authors approved the final version of the manuscript.

Acknowledgements

The authors thank the CLL patients who donated samples for this study, and Dr. Elías Campo (Pathology Laboratory, Hospital Clinic, Barcelona, Spain) for the generous gift of CLL and control tissue samples. This work was supported by grants SAF2015-69180R from the Ministerio de Economía y Competitividad (Spain)/Fondo Europeo de Desarrollo Regional (FEDER); S2010/BMD-2314 from the Comunidad de Madrid/European Union (AGP.); and by the Concerted Research Actions (KU Leuven C1 Grant C16/17/010) and the Research Foundation of Flanders (FWO-Vlaanderen, to EUB, PEVdS, and GO).

References

[1] T. Zenz, D, Mertens, R. Kuppers R, et al., From pathogenesis to treatment of chronic lymphocytic leukaemia, Nat. Rev. Cancer 10 (2010) 37–50.

[2] M. Hallek, T.D. Shanafelt, B. Eichhorst, Chronic lymphocytic leukaemia, Lancet 391 (2018) 1524–1537.

[3] T. Hacken, J.A. Burger, Microenvironment interactions and B-cell receptor signaling in Chronic Lymphocytic Leukemia: Implications for disease pathogenesis and treatment, Biochim. Biophys. Acta 1863 (2016) 401-413.

[4] A.R. Kini, N.E. Kay, L.C. Peterson, Increased bone marrow angiogenesis in B cell chronic lymphocytic leukemia, Leukemia 14 (2000) 1414-1418.

[5] S. Molica, A. Vacca, D. Ribatti, et al., Prognostic value of enhanced bone marrow angiogenesis in early B-cell chronic lymphocytic leukemia, Blood 100 (2002) 3344-3351.

[6] N.E. Kay, N.D. Bone, R.C. Tschumper, et al., B-CLL cells are capable of synthesis and secretion of both pro- and anti-angiogenic molecules. Leukemia 16 (2002) 911-919.

[7] Y. Xia, R.N. Lu, J. Li, Angiogenic factors in chronic lymphocytic leukemia. Leuk. Res. 36 (2012) 1211-1217.

[8] R. Maffei, S. Martinelli, I. Castelli, et al., Increased angiogenesis induced by chronic lymphocytic leukemia B cells is mediated by leukemia-derived Ang2 and VEGF, Leuk. Res. 34 (2010) 312-321.

[9] T.D. Shanafelt, N.E. Kay, The Clinical and Biologic Importance of Neovascularization and Angiogenic Signaling Pathways in Chronic Lymphocytic Leukemia, Semin. Oncol. 33 (2006) 174-185.

[10] N.E. Kay, T.D. Shanafelt, A.K. Strege, et al., Bone biopsy derived marrow stromal elements rescue chronic lymphocytic leukemia B-cells from spontaneous and drug induced cell death and facilitates an "angiogenic switch", Leuk. Res. 31 (2007) 899-906.

[11] J. Edelmann, L. Klein-Hitpass, A. Carpinteiro, et al., Bone marrow fibroblasts induce expression of PI3K/NF-kappaB pathway genes and a pro-angiogenic phenotype in CLL cells, Leuk. Res. 32 (2008) 1565-1572.

[12] R. Maffei, S. Fiorcari, J. Bulgarelli, et al., Physical contact with endothelial cells through β1- and β2- integrins rescues chronic lymphocytic leukemia cells from spontaneous and drug-induced apoptosis and induces a peculiar gene expression profile in leukemic cells, Haematologica 97 (2012) 952–960.

[13] G. Bergers, R. Brekken, G. McMahon, et al., Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis, Nat. Cell. Biol. 2 (2000) 737-744.

[14] J.E. Rundhaug, Matrix metalloproteinases and angiogenesis, J. Cell. Mol. Med. 9 (2005) 267-285.

[15] E.I. Deryugina, J.P. Quigley, Tumor angiogenesis: MMP-mediated induction of intravasation- and metastasis-sustaining neovasculature, Matrix Biol. 44-46 (2015) 94-112.

[16] B. Bauvois, J. Dumont, C. Mathiot, J.P. Kolb, Production of matrix metalloproteinase-9 in early stage B-CLL: suppression by interferons, Leukemia 16 (2002) 791-798.

[17] J. Redondo-Muñoz, E. Escobar-Díaz, R. Samaniego, et al., MMP-9 in B-cell chronic lymphocytic leukemia is up-regulated by alpha4beta1 integrin or CXCR4 engagement via distinct signaling pathways, localizes to podosomes, and is involved in cell invasion and migration. Blood 108 (2006) 3143-3151.

[18] E. Ugarte-Berzal, J. Redondo-Muñoz, P. Eroles, et al., VEGF/VEGFR2 interaction down-regulates matrix metalloproteinase-9 via STAT1 activation and inhibits B chronic lymphocytic leukemia cell migration, Blood 115 (2010) 846-849.

[19] N. Aguilera-Montilla, E. Bailón, R. Uceda-Castro, et al., MMP-9 affects gene expression in chronic lymphocytic leukemia revealing CD99 as an MMP-9 target and a novel partner in malignant cell migration/arrest, Oncogene 38 (2019) 4605-4619.

[20] B.S. Hanna, S.Öztürk, M. Seiffert, Beyond bystanders: Myeloid cells in chronic lymphocytic leukemia. Mol. Immunol. 110 (2019) 77-87.

[21] Z. Lopez-Dee, K. Pidcock, L.S. Gutierrez, Thrombospondin-1: multiple paths to inflammation. Mediators Inflamm. 2011 (2011) 296069.

[22] J. Redondo-Muñoz, E. Ugarte-Berzal, J.A. García-Marco, et al., $\alpha 4\beta 1$ integrin and 190-kDa CD44v constitute a cell surface docking complex for gelatinase B/MMP-9 in chronic leukemic but not in normal B cells. Blood 112 (2008)169-178.

[23] J. Redondo-Muñoz, E. Ugarte-Berzal, M.J. Terol, et al., Matrix metalloproteinase-9 promotes chronic lymphocytic leukemia B cell survival through its hemopexin domain. Cancer Cell 17 (2010) 160-172.

[24] Z. Chen, Z.C. Han. STAT3: a critical transcription activator in angiogenesis. Med. Res. Rev. 28 (2008) 185-200.

[25] Y.M. Oh, J.K. Kim, Y. Choi, et al., Prediction and Experimental Validation of Novel STAT3 Target Genes in Human Cancer Cells. PLoS One 4 (2009) e6911.

[26] X. Badoux, C. Bueso-Ramos, D. Harris, et al., Cross-talk between chronic lymphocytic leukemia cells and bone marrow endothelial cells: role of signal transducer and activator of transcription 3. Human Pathology 42 (2011) 1989–2000.

[27] M.H.A. van Attekum, E. Eldering, A.P. Kater. Chronic lymphocytic leukemia cells are active participants in microenvironmental cross-talk. Haematologica 102 (2017) 1469-1476.

[28] L.M. Aguirre Palma, H. Flamme, I. Gerke, K.A. Kreuzer. Angiopoietins Modulate Survival, Migration, and the Components of the Ang-Tie2 Pathway of Chronic Lymphocytic Leukaemia (CLL) Cells In Vitro. Cancer Microenviron. 9 (2016) 13-26.

[29] R. Maffei,S. Martinelli, R. Santachiara, et al., Angiopoietin-2 plasma dosage predicts time to first treatment and overall survival in chronic lymphocytic leukemia. Blood 116 (2010) 584-592.

[30] N.E. Sounni, C. Roghi, V. Chabottaux, et al., Up-regulation of vascular endothelial growth factor-A by active membrane-type 1 matrix

metalloproteinase through activation of Src-tyrosine kinases. J. Biol. Chem. 279 (2004) 13564-74.

[31] R. Roy, A. Dagher, C. Butterfield C, M.A.Moses. ADAM12 Is a Novel Regulator of Tumor Angiogenesis via STAT3 Signaling. Mol. Cancer Res. 15 (2017) 1608-1622.

Figure legends

Figure 1. MMP-9 is present in CLL tissues and enhances the CLL CM-induced endothelial cell proliferation. (A-B) Macrophages were differentiated to GM-MØ or M-MØ in the presence or absence of CLL CM. MMP-9 expression was analyzed at the indicated times in both types of MØ by qPCR (A) and Western blotting (B). (C) Immunofluorescence analysis of MMP-9 (green) and CD68 (MØ marker, red) expression in CLL LN and BM samples and in control non-CLL LN. Colocalization of MMP-9 and CD68 was observed. Cell nuclei were stained with DAPI (blue). Representative cases from four CLL LN, two CLL BM, and two control samples are shown. (D-E) CLL cells (5-10 x 10^6) were incubated with 110 nM MMP-9 or 0.5% BSA for 24 h, in RPMI, 005% FBS. (D) Cells were lysed and MMP-9 mRNA expression was analyzed by qPCR. Values represent the average of the 12 patients studied, after normalizing control cell values to 1. (E) ELISA analysis of MMP-9 production in the CM of 7 randomly selected samples among the ones used in (D). Medium alone, incubated on MMP-9 or BSA for 24 h was used as control (No cells). (F) The CM of MMP-9- or BSA-treated CLL cells (MMP-9-CM, BSA-CM) was added to HUVEC cultures and viability determined after 24 h by MTT. Control values were normalized to 100. *p≤0.05, **p≤0.01, ***p≤0.001.

Figure 2. MMP-9 induces a pro-angiogenic phenotype in CLL cells. 5-10 x 10^6 CLL cells were cultured on immobilized MMP-9 or BSA for 24 or 48 h. Cells were lysed and the expression of the indicated genes (A) and proteins (B) was analyzed by qPCR (A) or Western blotting (B), respectively. Values of BSA-treated cells were normalized to 1. *p≤0.05, **p≤0.01, ***p≤0.001.

.

Figure 3. Downregulation of *TSP-1* by MMP-9 involves $\alpha4\beta1$ integrin, SFK activity and STAT3. (A) Efficiency of the transfection of CLL cells (15 x 10^6) with two different $\alpha4$ siRNAs, measured by qPCR after 48 h. Values for control siRNA-transfected cells were normalized to 1. (B) *TSP-1* expression in $\alpha4$ siRNA-transfected cells, treated or not with MMP-9 for 24 h. (C) *TSP-1* expression in CLL cells pre-treated for 1 h with the indicated inhibitors (all at 5 μM except for Saracatinib: 1 μM) or vehicle (Ctrol) and incubated on MMP-9 or BSA for 24 h, in the presence of inhibitors. (D) *STAT3* expression in CLL cells transfected with two different *STAT3* siRNAs for 48 h. (E) *TSP-1* expression in STAT3 siRNA-transfected cells after treatment with MMP-9 or BSA for 24 h. In panels B, C and E, values for control siRNA-transfected cells cultured on MMP-9 were normalized to 1. Ctrol, control. *p≤0.05, ***p≤0.001.

Table 1.
Clinical characteristics of CLL patients

Patients	Sex/Age	Stage[a]	Ig status[a]	Alpha4[b]	Beta1[b]	MMP-9[b]
1	M/79	B/II	UM	30.1	95.7	6.6
2	M/72	C/IV	UM	48.9	54.3	4.7
3	F/69	C/III	UM	99	97.2	1.7
4	F/70	C/IV		80.7	78.2	Nd
5	M			78.5	99.5	9.1
6	M/69	C/IV		37	72.7	15.8
7	F/67			97.1	99.3	1.5
8	F/58	B/II	MUT	99.3	98.8	11.8
9	M		MUT	88.5	98.4	14.5
10	M/48	B/I	UM	48.8	66.1	5
11	M			94.1	98	Nd
12	M/62	B/I	MUT	89.7	97.8	11.5
13	F/55	B/II	MUT	98.7	97	7.7
14	M/82	C/IV	MUT	94.1	95	Nd
15	M/47	B/II	MUT	78.3	95.8	4.5
16	M/75	B/I	UM	75.7	73.9	12.6
17	M/50	B/II	MUT	36.3	69.1	2.8
18	M/70	B/II	UM	98.3	91.6	2.7
19	F/83	B/II	MUT	77.6	82.7	7.1
20	M			99.5	53.3	17.5
21	F/58	B/II	UM	41.2	36.1	6.1
22	M/63	B/II	MUT	47.9	28.1	15.9
23	F/47	B/II	UM	43.9	3.8	21.6
24	F/84	A/I	UM	37	43.8	6
25	F/83	C/IV	UM	94.4	99.7	7.3
26	M/67	B/II	MUT	39.8	29.1	2.9
27	F/88	B/II	UM	17.6	20.5	20.1
28	M/70	B/II	UM	51.9	44.1	3.5
29	M/80	B/II	UM	97.1	94.6	5.4
30	M/66	B/II	UM	45.2	25.3	12.3

[a]Clinical characteristics according to Ref. [1,2]. [b]Values represent the percentage of positive cells. F, female. M, male. UM, unmutated Ig. MUT, mutated Ig. Nd, not determined.

Table 2
Oligonucleotide sequences used in this study.

	Gene name	Oligonucleotide sequence
Primer	*VEGF*	5′-GCAGCTTGAGTTAAACGAACG-3′ 5′-GGTTCCCGAAACCCTGAG-3′
	MMP-9	5′-GAACCAATCTCACCGACAGG-3′ 5′-GCCACCCGAGTGTAACCATA-3′
	TSP-1	5′-CAATGCCACAGTTCCTGATG -3′ 5′-TGGAGACCAGCCATCGTC -3′
	ANG2	5′-TGCAAATGTTCACAAATGCTAA -3′ 5′-AAGTTGGAAGGACCACATGC -3′
	bFGF	5′-GGCTTCTTCCTGCGCATCC -3′ 5′-GGTAACGGTTAGCACACACTCCTT-3′
	$\alpha 4$	5′-GATGAAAATGAGCCTGAAA-3′ 5′-GCCATACTATTGCCAGTGT-3′
	STAT3	5′-TCCTGAAGCTGACCCAGGTA-3′ 5′-GGCAGGTCAATGGTATTGCT-3′
	TBP	5′-CGGCTGTTTAACTTCGCTTC-3′ 5′-CACACGCCAAGAAACAGTGA-3′
siRNA	$\alpha 4_1$	5’-CUGAAACGUGCAUGGUGGAdTdT-3’
	$\alpha 4_2$	5’-GAACUUAACUUUCCAUGUUdTdT-3’
	$STAT3_1$	5’-GGCUGGACAAUAUCAUUGAdTdT-3’
	$STAT3_2$	5’-GCCUCAAGAUUGACCUAGAdTdT-3’
	Control	5’-AUUGUAUGCGAUCGCAGACdTdT-3’

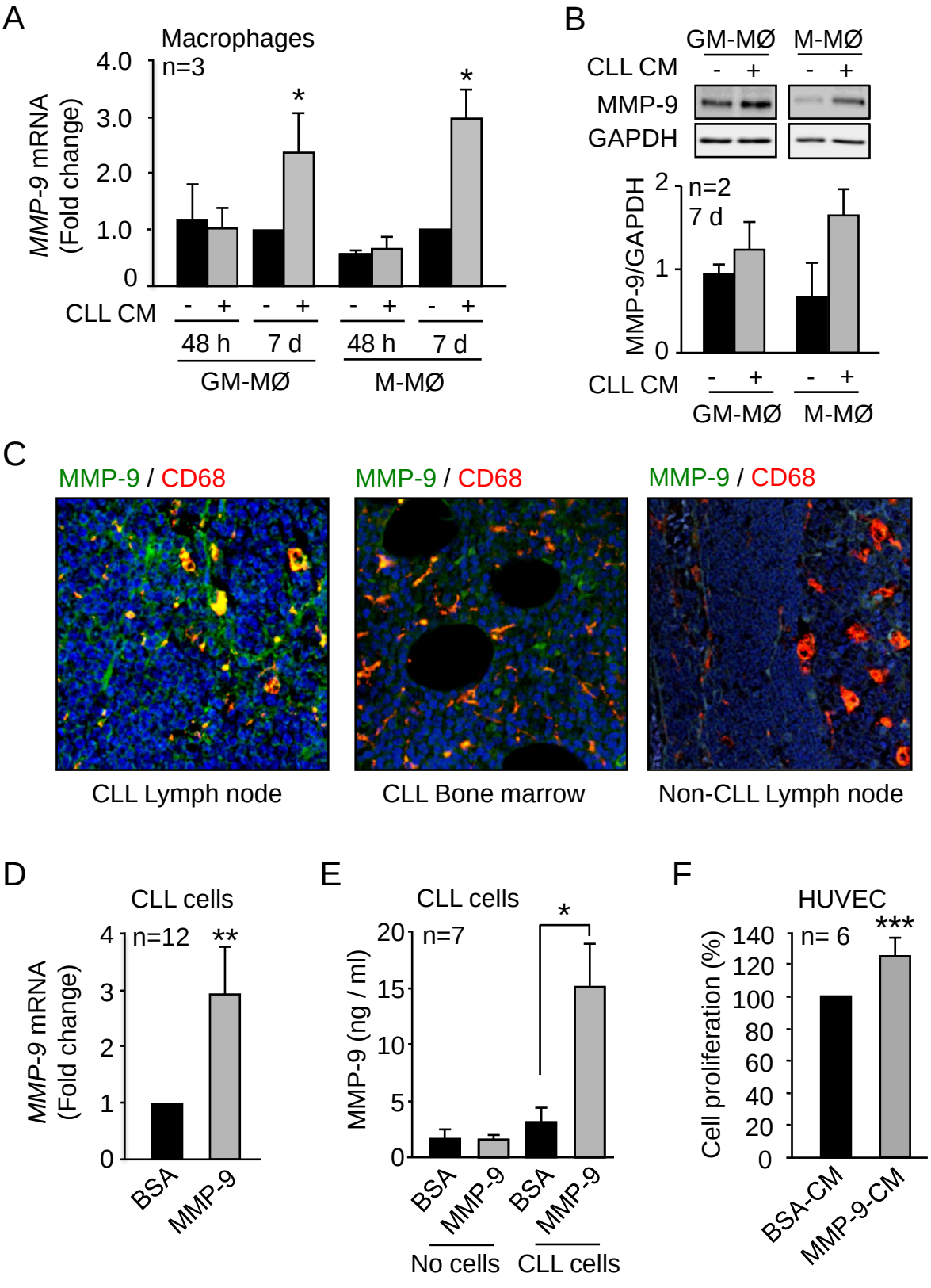
A
Macrophages
n=3
MMP-9 mRNA (Fold change)
4.0
3.0
2.0
1.0
0
*
*
CLL CM
- + - + - + - +
48 h 7 d 48 h 7 d
GM-MØ M-MØ
B
GM-MØ M-MØ
CLL CM - + - +
MMP-9
GAPDH
MMP-9/GAPDH
2
1
0
n=2
7 d
CLL CM - + - +
GM-MØ M-MØ
C
MMP-9 / CD68
MMP-9 / CD68
MMP-9 / CD68
CLL Lymph node
CLL Bone marrow
Non-CLL Lymph node
D
CLL cells
n=12
**
MMP-9 mRNA (Fold change)
4
3
2
1
0
BSA
MMP-9
E
CLL cells
n=7
*
MMP-9 (ng / ml)
20
15
10
5
0
BSA
MMP-9
BSA
MMP-9
No cells
CLL cells
F
HUVEC
n= 6
***
Cell proliferation (%)
140
120
100
80
60
40
20
0
BSA-CM
MMP-9-CM

Figure 1

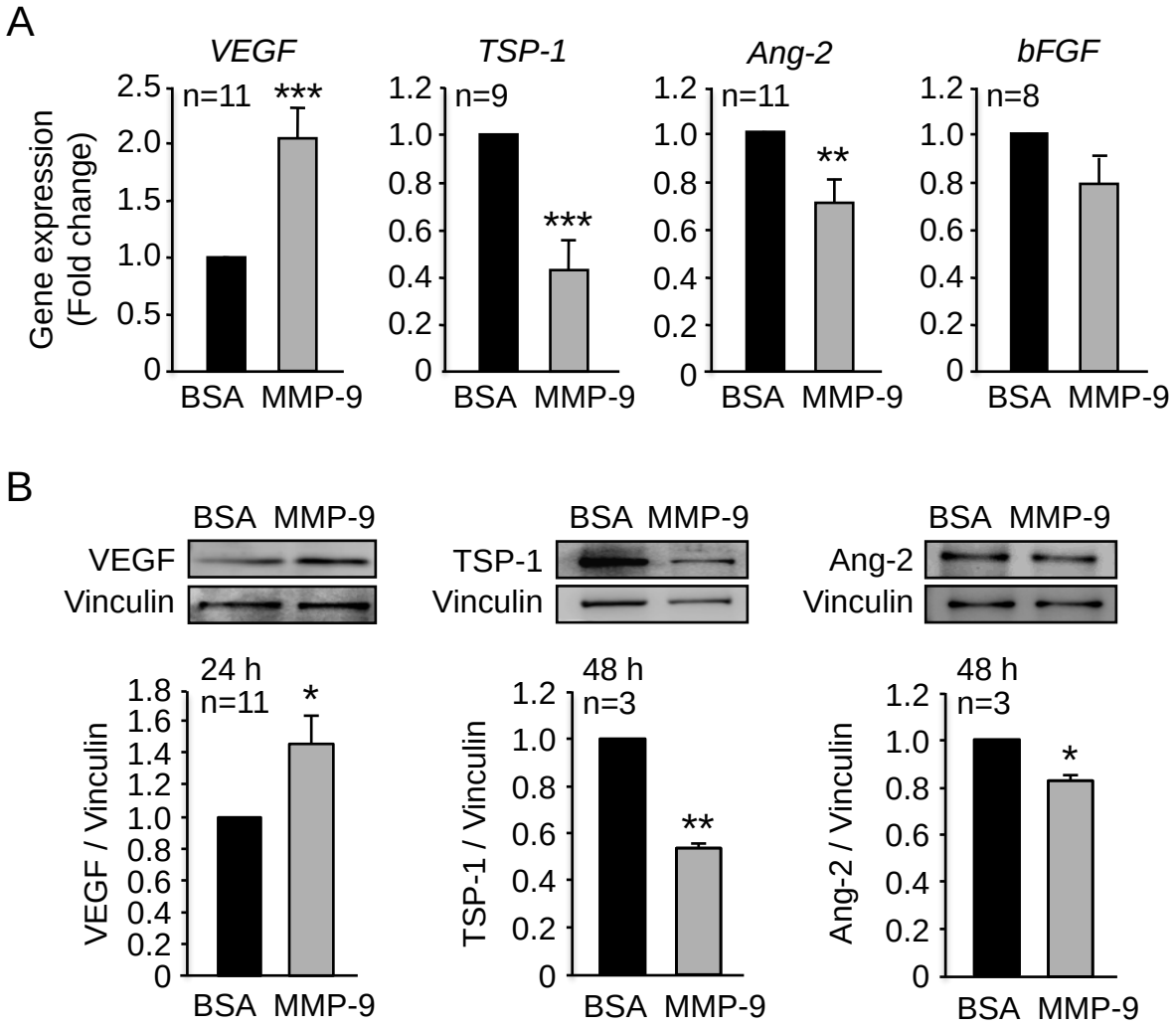
A
VEGF
TSP-1
Ang-2
bFGF
Gene expression (Fold change)
n=11
***
n=9
***
n=11
**
n=8
BSA
MMP-9
B
BSA MMP-9
VEGF
Vinculin
TSP-1
Vinculin
Ang-2
Vinculin
24 h
n=11
*
48 h
n=3
**
48 h
n=3
*
VEGF / Vinculin
TSP-1 / Vinculin
Ang-2 / Vinculin

Figure 2

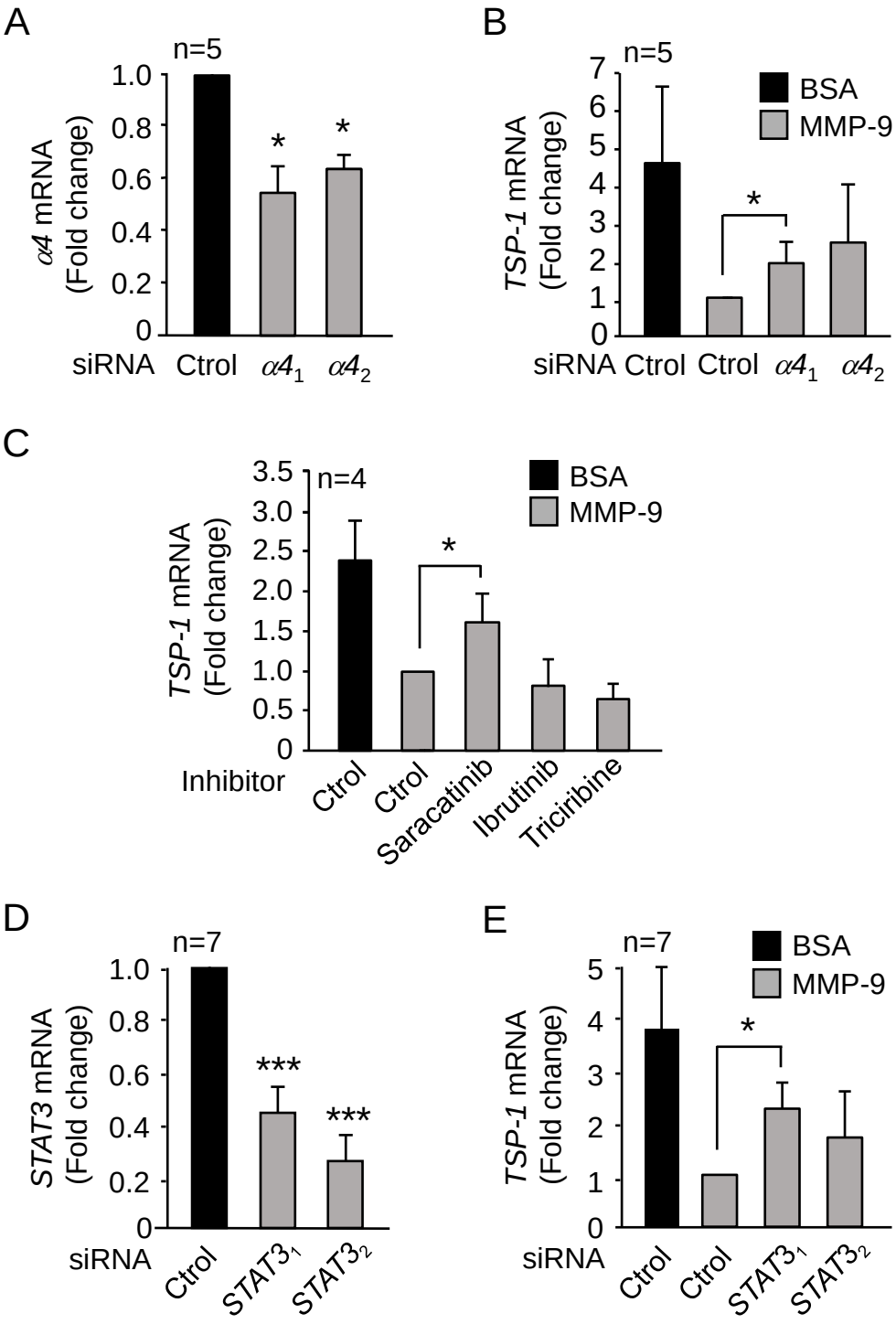
A
n=5
α4 mRNA (Fold change)
1.0
0.8
0.6
0.4
0.2
0
*
*
siRNA Ctrol α4₁ α4₂
B
n=5
TSP-1 mRNA (Fold change)
7
6
5
4
3
2
1
0
BSA
MMP-9
*
siRNA Ctrol Ctrol α4₁ α4₂
C
n=4
TSP-1 mRNA (Fold change)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
BSA
MMP-9
*
Inhibitor
Ctrol
Ctrol
Saracatinib
Ibrutinib
Triciribine
D
n=7
STAT3 mRNA (Fold change)
1.0
0.8
0.6
0.4
0.2
0
***
***
siRNA
Ctrol
STAT3₁
STAT3₂
E
n=7
TSP-1 mRNA (Fold change)
5
4
3
2
1
0
BSA
MMP-9
*
siRNA
Ctrol
Ctrol
STAT3₁
STAT3₂

Figure 3

Supplemental Material

Matrix metalloproteinase-9 induces a pro-angiogenic profile in chronic lymphocytic leukemia cells

Noemí Aguilera-Montilla[a], Elvira Bailón[a], Estefanía Ugarte-Berzal[b], Rebeca Uceda-Castro[a], María Prieto-Solano[a], Elena García-Martínez[c], Rafael Samaniego[c], Philippe E. Van den Steen[b], Ghislain Opdenakker[b], José A. García-Marco[d], and Angeles García-Pardo[a*]

Supplemental Methods

1. Antibodies and reagents

Monoclonal antibodies (mAbs) to α4 (HP2/1) and β1 (TS2/16) integrin subunits were from Dr. F. Sánchez-Madrid (Hospital de la Princesa, Madrid, Spain). mAbs against MMP-9 (sc-21733), TSP-1 (sc-59887), Ang-2 (sc-74403), and GAPDH (sc-32233) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). mAb anti-VEGF (VG-1) was from eBioscience (San Diego, CA, USA). mAb anti-CD68 (PG-M1) was from Dako Denmark A/S (Glostrup, Denmark). mAb anti-vinculin (V9131) and Saracatinib (Src kinase family (SFK) inhibitor) were from Sigma-Aldrich (Saint Louis, MI, USA). Isotype control mouse IgG1 was from BD Pharmingen (Franklin Lakes, NJ, USA). Ibrutinib (Bruton´s tyrosine kinase (BTK) inhibitor) was from Selleckchem (Munich, Germany). Triciribine (Akt inhibitor) was from Calbiochem (San Diego, CA, USA). Recombinant MMP-9 was prepared as reported [1].

2. Flow cytometry

2 x 10^5 primary CLL cells were incubated (30 min, 4ºC) in 100 µl PBS/1%BSA with appropriate primary antibodies or isotype controls, washed and incubated

(30 min, 4ºC) with Alexa 488-labeled secondary Abs (Jackson ImmunoResearch, Cambridgeshire, UK). Samples were analyzed on a Cytomics FC500 or a Coulter Epics XL flow cytometer (Beckman Coulter, Fullerton, CA).

3. Western blotting

5-10 x 10^6 CLL cells were lysed (30 min, 4ºC) in ice-cold 20 mM Tris-HCl pH 7.5, 137 mM NaCl, 10% glycerol, 1% NP-40, 1 mM NaF, 1 mM Na_3VO_4, and protease/phosphatase inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Protein concentration was determined by the Pierce™ BCA Protein Assay Kit (Thermo Scientific, Whalthman, MA, USA). Lysates were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). After electrophoresis, membranes were blocked with 5% BSA for 1 h and incubated (4ºC, 16 h) with primary Abs, followed by incubation for 1 h at room temperature with HRP-labelled secondary Abs. Protein bands were developed using the enhanced chemiluminiscent detection method (GE Healthcare Europe GmbH, Barcelona, Spain) and quantitated using the ImageJ program [2]. Protein load was corrected using vinculin as internal standard.

4. ELISA assays

5 x 10^6 CLL cells in RPMI, 0.05% FBS were cultured on immobilized MMP-9 (110 nM) or BSA (0.5%) for 24 h. The conditioned medium was collected and the amount of secreted MMP-9 was measured by ELISA (Quantikine ELISA, R&D Systems, Minneapolis, MN, USA), following the manufacturer´s protocol. Medium without cells was also added to MMP-9 or BSA-coated wells for 24 h and used as a control.

5. Statistical analyses

Statistical significance of the data was determined using the two-tailed Student's t-test. A p value of ≤0.05 was considered significant. Analyses were performed using Microsoft Excell (Microsoft Co, Redmond, WA, USA). All values are expressed as means ± standard error.

References

[1] P.E. Van den Steen, I. Van Aelst, V. Hvidberg, et al., The hemopexin and O-glycosylated domains tune gelatinase B/MMP-9 bioavailability via inhibition and binding to cargo receptors. J. Biol. Chem. 281 (2006) 18626-18637.

[2] C.A. Schneider, W.S. Rasband, K.W. Eliceiri. NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9 (2012) 671-675.