

# Matrix Metalloproteinase-9 Promotes Chronic Lymphocytic Leukemia B Cell Survival through Its Hemopexin Domain

Javier Redondo-Muñoz,<sup>1</sup> Estefanía Ugarte-Berzal,<sup>1</sup> María José Terol,<sup>2</sup> Philippe E. Van den Steen,<sup>3</sup> Mercedes Hernández del Cerro,<sup>1</sup> Martin Roderfeld,<sup>4</sup> Elke Roeb,<sup>4</sup> Ghislain Opdenakker,<sup>3</sup> José A. García-Marco,<sup>5</sup> and Angeles García-Pardo<sup>1,\*</sup>

<sup>1</sup>Cellular and Molecular Medicine Programme, Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, E-28040 Madrid, Spain

<sup>2</sup>Servicio de Hematología y Medicina Oncológica, Hospital Clínico Universitario, E-64010 Valencia, Spain

<sup>3</sup>Rega Institute for Medical Research, University of Leuven, B-3000 Leuven, Belgium

<sup>4</sup>Department of Gastroenterology, Justus Liebig University, G-35390 Giessen, Germany

<sup>5</sup>Servicio de Hematología, Hospital Universitario Puerta de Hierro, E-28222 Madrid, Spain

\*Correspondence: [agarciapardo@cib.csic.es](mailto:agarciapardo@cib.csic.es)

DOI 10.1016/j.ccr.2009.12.044

## SUMMARY

Matrix metalloproteinase-9 (MMP-9) is the major MMP produced by B-CLL cells and contributes to their tissue infiltration by degrading extracellular and membrane-anchored substrates. Here we describe a different function for MMP-9 in B-CLL, which involves the hemopexin domain rather than its catalytic function. Binding of soluble or immobilized (pro)MMP-9, a catalytically inactive proMMP-9 mutant, or the MMP-9 hemopexin domain to its docking receptors  $\alpha 4\beta 1$  integrin and CD44v, induces an intracellular signaling pathway that prevents B-CLL apoptosis. This pathway is induced in all B-CLL cases, is active in B-CLL lymphoid tissues, and consists of Lyn activation, STAT3 phosphorylation, and Mcl-1 upregulation. Our results establish that MMP/receptor binding induces intracellular survival signals and highlight the role of (pro)MMP-9 in B-CLL pathogenesis.

## INTRODUCTION

Matrix metalloproteinase-9 (MMP-9; gelatinase B), a member of the MMP enzyme family, is widely expressed in many tissues and cell types, including those of lymphoid lineage (Opdenakker et al., 2001; Stamenkovic, 2003; Deryugina and Quigley, 2006). Like other members of the family, MMP-9 mainly degrades components of the extracellular matrix, but it may also cleave proteins involved in cell migration/invasion, apoptosis/cell survival, and angiogenesis as well as intracellular proteins. (Van den Steen et al., 2002; Egeblad and Werb, 2002; Cauwe et al., 2007; Xu et al., 2008). On the basis of extensive data from biological, functional gene knockout, and clinicopathological studies on enzyme activities, MMP-9 has been defined as an exquisite target in inflammatory and vascular diseases (Hu et al., 2007).

MMP-9 is the major MMP found in certain B cell malignancies such as B cell chronic lymphocytic leukemia (B-CLL). B-CLL cells produce mostly 92 kDa proMMP-9 (Bauvois et al., 2002; Redondo-Muñoz et al., 2006) and elevated intracellular levels of this MMP correlate with advanced stage and poor patient survival (Kamiguti et al., 2004), suggesting a role for MMP-9 in B-CLL pathogenesis. B-CLL is characterized by the accumulation in the peripheral blood (PB) of CD5<sup>+</sup> B lymphocytes, which progressively infiltrate the bone marrow (BM) and secondary lymphoid tissues (Chiorazzi et al., 2005; Caligaris-Cappio and Hamblin, 2008). B-CLL cells are resistant to apoptosis, and several molecules including the chemokine receptors CXCR4 and CCR7, vascular endothelial factor receptors, and  $\alpha 4\beta 1$  integrin have been shown to participate in B-CLL cell migration and to provide survival signals, thus contributing to B-CLL

### Significance

The local production of MMPs in the tumor environment by cancer or bystander cells has been associated with invasive or metastatic phenotypes and was a preamble for many studies on inhibitors of MMP catalysis for cancer therapy. Here we demonstrate that MMP-9 and specifically its hemopexin domain induce signals for B-CLL cell survival by a noncatalytic mechanism. This finding not only constitutes a new mechanism to account for the failure of catalytic MMP inhibitors in cancer treatment but it also defines hemopexin domains as potential cancer targets and opens new directions for B-CLL treatment.

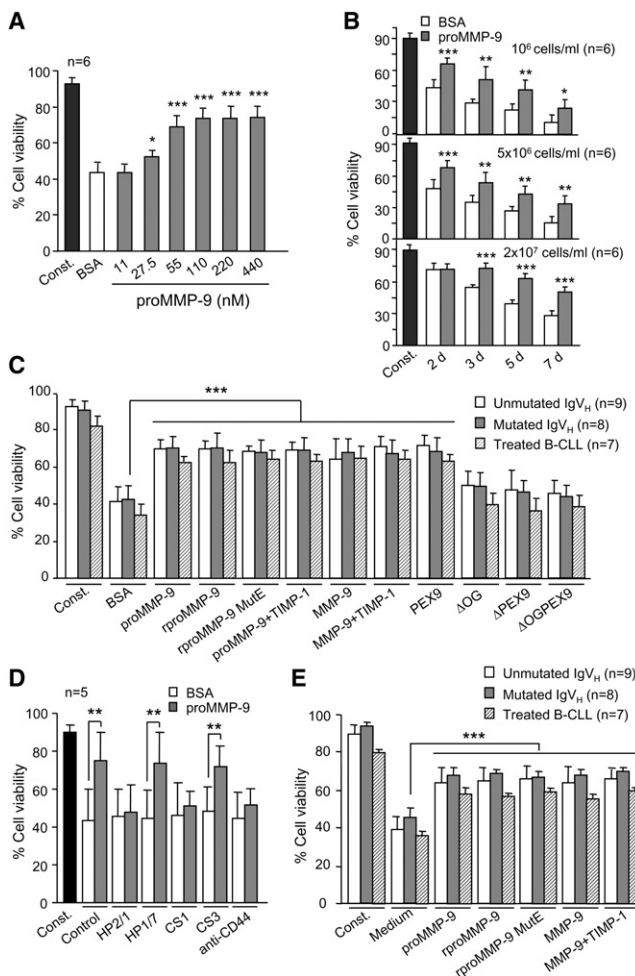
pathology (Alfonso-Pérez et al., 2006; Burger et al., 2000; De la Fuente et al., 1999, 2002; Till et al., 2002, 2005). We and others previously reported that pro and mature MMP-9 [(pro)MMP-9] also play an important role in B-CLL transendothelial migration and basement membrane invasion (Kamiguti et al., 2004; Redondo-Muñoz et al., 2006, 2008a). Additionally, MMP-9 was involved in the survival effect induced by B-CLL cell interaction with BM stromal cells (Ringshausen et al., 2004). In both cases, cell migration and cell survival, the effect of MMP-9 was due to its proteolytic activity.

Although (pro)MMP-9 is a secreted protease and does not bind to normal B cells (Redondo-Muñoz et al., 2008b), it has been consistently detected at the B-CLL cell surface (Kamiguti et al., 2004; Redondo-Muñoz et al., 2006, 2008b). Targeting MMP-9 to the cell surface is mediated by specific docking receptors and is thought to localize and enhance its proteolytic activity at the pericellular space (Yu and Stamenkovic, 2000; Van den Steen et al., 2002; Fridman et al., 2003). We have recently shown that  $\alpha 4\beta 1$  integrin and a 190 kDa CD44 variant (CD44v) constitute a docking complex for (pro)MMP-9 at the B-CLL cell surface and that the MMP-9 hemopexin domain (PEX9) is required for this interaction (Redondo-Muñoz et al., 2008b). Binding of (pro)MMP-9 to this complex results in inhibition of B-CLL cell migration and this again requires MMP-9 proteolytic activity. These previous reports suggest an important role for cell-associated MMP-9 in the regulation of B-CLL cell migration and arrest. In the present study, we have investigated whether cell-bound MMP-9 may influence B-CLL cell survival and whether this involves MMP-9 catalytic activity.

## RESULTS

### Adhesion to proMMP-9 or Binding of Soluble proMMP-9 Via $\alpha 4\beta 1$ Integrin and CD44v Protects B-CLL Cells Against Spontaneous Apoptosis

We studied whether purified proMMP-9 influenced the spontaneous apoptosis of cultured B-CLL cells. In initial experiments, six B-CLL samples were cultured at  $10^6$  cells/ml on plates coated with BSA or various concentrations of proMMP-9 and viability was measured by flow cytometry after 48 hr. Figure 1A shows that, starting at 27.5 nM, proMMP-9 significantly increased B-CLL cell viability compared to BSA, reaching a nearly maximal effect at 55 nM. A 55–60 nM concentration was chosen for subsequent studies. To assess the time and cell density dependence of proMMP-9 action, we studied cell cultures for time intervals between 1 and 7 days and concentrations ranging from  $10^6$  to  $2 \times 10^7$  cells/ml. No differences between BSA and proMMP9-treated cells were observed after 1 day for any of the conditions used (data not shown). However, at day 2 ( $10^6$  or  $5 \times 10^6$ /ml) or day 3 ( $10^7$ /ml [data not shown] or  $2 \times 10^7$ /ml), cells cultured on proMMP-9 showed significantly higher viability than cells on BSA (Figure 1B). Thus, increasing the cell density delayed the onset of apoptosis, but once this started, proMMP-9 had a clear survival effect regardless of the cell concentration. Although the viability of all tested cell cultures decreased with time, a significant survival effect by proMMP-9 persisted for the 7 days of the assay for all conditions (Figure 1B). For subsequent studies,  $10^6$  cells/ml concentration and 2 day cultures were chosen.



**Figure 1. proMMP-9/Receptor Interaction Induces B-CLL Cell Survival**

(A) B-CLL cells ( $10^6$ /ml) were incubated on 96-well plates coated with 0.5% BSA or the indicated concentrations of proMMP-9 for 48 hr. Cell viability was determined by flow cytometry using Annexin V and propidium iodide. Const. (constitutive) represents the average viability of fresh B-CLL cells. (B) B-CLL cells at the indicated concentrations were incubated on BSA or 60 nM proMMP-9 for the indicated times and average viability values are shown. (C) B-CLL cells ( $10^6$ /ml) were cultured on BSA or the indicated proteins (all at 60 nM) and viability was measured after 48 hr. (D) B-CLL cells with or without previous incubation with Abs or peptides were added to BSA- or proMMP-9-coated wells and cell viability was determined after 48 hr. (E) B-CLL cells from the same subgroups shown in (C) were incubated in suspension with the indicated proteins and viability was measured after 48 hr. Average values  $\pm$  SD are shown. n indicates the number of patient samples analyzed. \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ . See also Figure S1.

To determine whether the proMMP-9 survival function affected all B-CLL cases or was specific for a selected subtype, we analyzed B-CLL samples with mutated or unmutated IgV<sub>H</sub> genes separately. We also studied a group of previously treated B-CLL patients (Table 1), but with a prominent B-CLL cell population. Figure 1C shows that all three subgroups showed significantly higher viability (69.5%, 70%, and 62%, for unmutated,

**Table 1. Clinical Characteristics of B-CLL Patients**

Patient <sup>a</sup>	Sample	Sex/Age	Stage <sup>b</sup>	CD38 <sup>c</sup>	ZAP70 <sup>c</sup>	Ig Status <sup>c</sup>	Therapy
P1	PB	M/62	A	+	ND	Unmutated	None
P2	PB	M/75	A	+	+	Unmutated	None
P3	PB	M/77	A	–	–	Mutated	None
P4	PB	M/56	B/II	–	–	Mutated	None
P5	PB	M/63	B/II	ND	ND	ND	None
P6	PB	F/84	A	+	ND	Unmutated	None
P7	PB	M/64	B/II	+	ND	ND	None
P8	PB	M/76	A	+	ND	Mutated	None
P9	PB	M/36	A	–	ND	ND	None
P10	PB	F/75	B/II	+	+	ND	None
P11	PB	F/51	ND	–	–	Mutated	None
P12	PB	M/52	B/II	+	+	Unmutated	None
P13	PB	F/75	A	+	+	Mutated	None
P14	PB	M/60	A	–	–	Unmutated	None
P15	PB	F/44	A	+	+	Unmutated	None
P16	PB	M/78	B/II	–	ND	Mutated	None
P17	PB	F/71	A	–	–	Unmutated	None
P18	PB	M/67	B/II	–	–	Mutated	None
P19	PB	F/75	A	+	+	Unmutated	None
P20	PB	F/66	B/II	–	–	Mutated	None
P21	PB	F/68	B/II	+	–	Mutated	None
P22	PB	M/82	A	+	+	Unmutated	None
P23	PB	M/72	A/II	ND	–	Unmutated	Fludara
P24	PB	M/69	B/II	–	+	Mutated	Fludara + cyclophosphamide
P25	PB	F/71	A/II	+	+	Unmutated	Leukeran
P26	PB	F/68	B/II	+	–	Mutated	Fludara + cyclophosphamide
P27	PB	M/78	B/II	–	–	Unmutated	Fludara + cyclophosphamide
P28	PB	M/72	C/IV	+	–	Unmutated	Leukeran + rituximab
P29	PB	M/65	C/IV	–	–	Mutated	Alemtuzumab
P30	PB + BM	M/60	A/II	–	–	Mutated	None
P31	PB + BM	M/55	A/II	+	+	Unmutated	None
P32	PB + BM	M/60	C/IV	+	+	Mutated	None
P33	PB + BM	M/50	A/II	–	–	Unmutated	None
P34	PB + LN	M/57	A/II	–	+	Mutated	None
P35	PB + LN	M/65	B/II	+	+	Unmutated	None

The following abbreviations are used: ND, not determined; PB, peripheral blood; BM, bone marrow; LN, lymph nodes.

<sup>a</sup> Because the described analyses were done for the whole group of patients (P), for specific subgroups, or for individual samples, in all figures patient samples are indicated by the tabulated P numbers or by the total numbers (n =) from this cohort of 35 B-CLL patients.

<sup>b</sup> According to Rai et al. (1975) and Binet et al. (1981).

<sup>c</sup> The Ig status and the CD38 and ZAP70 expression in B-CLL cells has prognostic value (Chiorazzi et al., 2005; Caligaris-Cappio and Hamblin, 2008).

mutated, or treated samples, respectively) when cultured on proMMP-9 compared to their respective counterparts cultured on BSA (41%, 42%, and 34%). A similar significant ( $p \leq 0.001$ ) anti-apoptotic effect was observed on cells attached to recombinant full-length proMMP-9 (rproMMP-9; Figure 1C). Comparison of ZAP70<sup>+</sup>/CD38<sup>+</sup> (five samples) versus ZAP70<sup>–</sup>/CD38<sup>–</sup> (six samples) also rendered similar results (data not shown). To confirm these results we also measured cell viability by the MTT method. Parallel determinations with Annexin V/propidium iodide or MTT on six B-CLL samples (three with

unmutated and three with mutated IgV<sub>H</sub>) rendered identical results by both methods (Figures S1A and S1B, available online). Moreover, no cell proliferation was observed on these cultures, measured by cell counting and cycle analyses with propidium iodide (data not shown).

To establish whether MMP-9 proteolytic activity was involved in this survival function, we studied the effect of a rproMMP-9 protein carrying a mutation in the catalytic site (rproMMP-9MutE) and devoid of catalytic activity (Van den Steen et al., 2006). We previously showed that B-CLL cells attach to this

mutant as effectively as to proMMP-9 or natural proMMP-9 (Redondo-Muñoz et al., 2008b). As shown in Figure 1C, rproMMP-9MutE also protected B-CLL cells from spontaneous apoptosis, regardless of their IgV<sub>H</sub> mutational stage or the receipt of treatment. Moreover, blocking MMP-9 activity by previous incubation with its inhibitor TIMP-1 did not affect the observed anti-apoptotic effect (Figure 1C). In agreement with this, cell adhesion to activated 83 kDa MMP-9 rendered similar viability levels as adhesion to proMMP-9 and, as observed for the proform, this effect was not abolished by TIMP-1 (Figure 1C). To further confirm these results we studied the effect of several proMMP-9 truncated variants on B-CLL cell viability. Figure 1C shows that the mouse MMP-9 hemopexin domain (PEX9) (Roeb et al., 2002) also induced cell survival of all B-CLL samples and to a similar level as human proMMP-9. Removal of the PEX9 domain and/or the adjacent O-glycosylated region abolished cell adhesion to proMMP-9 (data not shown; Redondo-Muñoz et al., 2008b) and the anti-apoptotic effect (Figure 1C). To establish whether this function was unique for PEX9 or shared by other MMP hemopexin domains, we analyzed the effect of the human MMP-2 hemopexin region (PEX2). Culturing B-CLL cells (n = 8 patients, 5 with mutated and 3 with unmutated IgV<sub>H</sub>) on PEX2 for 48 hr resulted in significantly higher viability (66%) than culturing these on BSA (40%; data not shown), suggesting that the ability to induce cell survival may be a common property of MMP hemopexin domains.

To confirm that proMMP-9-induced viability was due to interaction with its surface receptors,  $\alpha 4 \beta 1$  integrin and CD44v, we blocked  $\alpha 4 \beta 1$  with the HP2/1 mAb or the CS-1 synthetic peptide or CD44 with specific Abs. These treatments prevented adhesion to proMMP-9 (data not shown) and the subsequent anti-apoptotic function, whereas the control anti- $\alpha 4$  mAb HP1/7 or the CS-3 peptide had no effect (Figure 1D).

Because proMMP-9 is a secreted protease, we next studied whether soluble proMMP-9 also produced a survival effect. B-CLL cells (same subgroups as in Figure 1C) were incubated in suspension with or without proMMP-9 and viability was measured after 48 hr. FACS analyses confirmed that proMMP-9 remained bound to the B-CLL cell surface at this time (Figure S1C) and this resulted in significantly higher cell viability compared to control cells (Figure 1E). As observed for immobilized proMMP-9, soluble rproMMP-9MutE or (pro)MMP-9 complexed with TIMP-1 also increased B-CLL viability (Figure 1E). Altogether these results established that binding of proMMP-9 to its docking receptors is necessary for prevention of apoptosis and that the antiapoptotic effect does not require MMP-9 proteolytic activity.

#### Cell Adhesion to proMMP-9 Upregulates Mcl-1 and Prevents Mitochondrial Damage and Caspase Activation

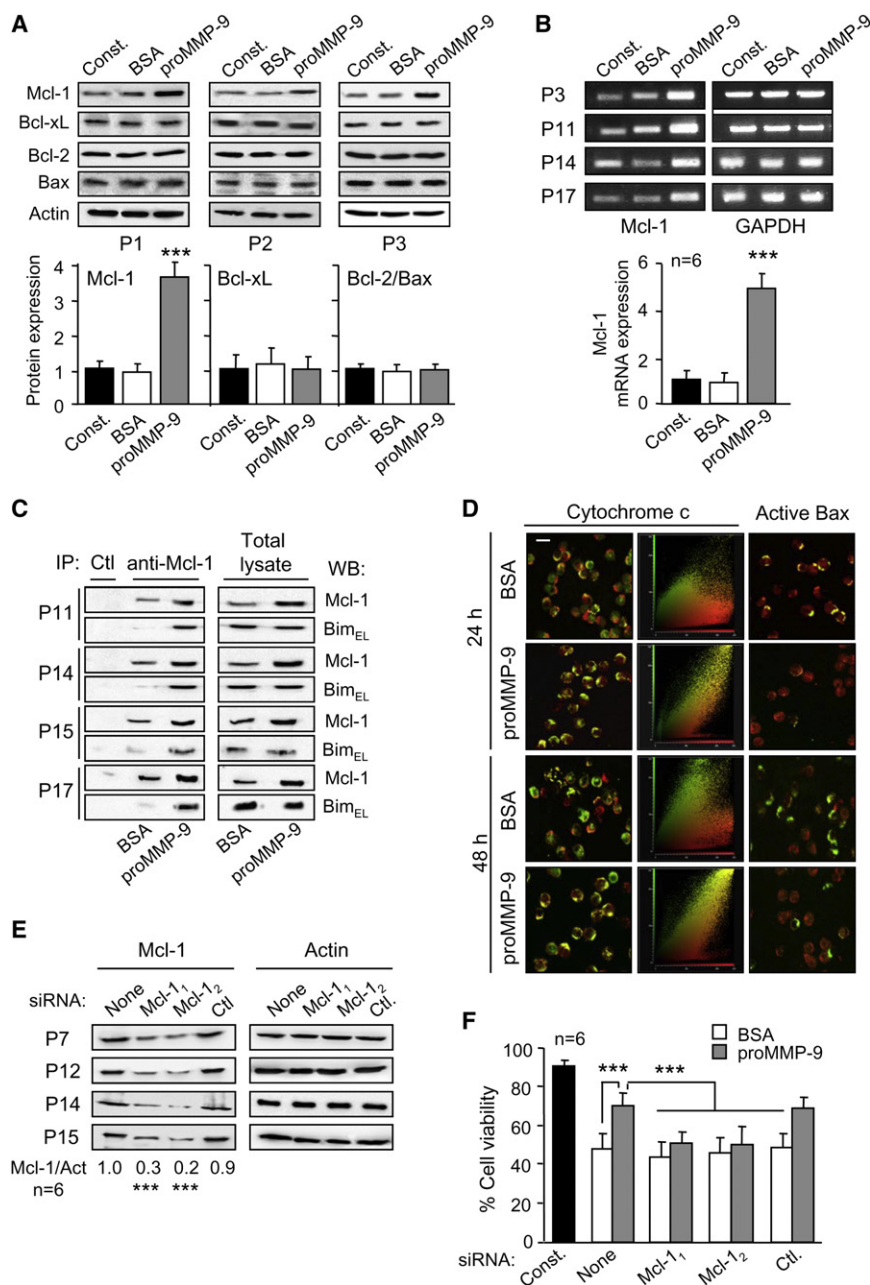
To analyze the antiapoptotic mechanism induced by proMMP-9, we first studied the possible involvement of Bcl-2 protein family members. B-CLL cells (n = 3) were incubated on BSA or proMMP-9 and after 48 hr were lysed and analyzed by western blotting. Figure 2A shows that Mcl-1, but not Bcl-xL or the Bcl-2/Bax ratio, was upregulated in all samples cultured on proMMP-9 (average 3.6-fold increases of Mcl-1 compared to constitutive values), but not on their corresponding BSA cultures. Mcl-1 protein degradation was not observed in these assays. Mcl-1

levels already increased 2.9-fold after 24 hr, in spite of a similar viability at this time between proMMP-9 and BSA cultures (Figure S2A). Soluble proMMP-9 and the immobilized PEX9 and PEX2 domains also specifically induced Mcl-1 upregulation (Figures S2B and S2C). Moreover, *Mcl-1* mRNA was also significantly ( $p \leq 0.001$ ) induced in B-CLL cells cultured for 2 hr on proMMP-9, compared to BSA-cultured cells (Figure 2B). Elevated *Mcl-1* mRNA levels were maintained for at least 24 hr (data not shown). Addition of actinomycin D after the 2 hr culture followed by RT-PCR analysis at various time points revealed that proMMP-9 did not induce *Mcl-1* mRNA stabilization (Figure S2D). These results established that proMMP-9 regulated Mcl-1 at the transcriptional level.

We next studied the role of Mcl-1 in the antiapoptotic pathway elicited by proMMP-9. Figure 2C shows that anti-Mcl-1 Abs pulled down the BH3-only proapoptotic protein Bim from lysates of proMMP-9 but not BSA-cultured B-CLL cells. This indicated that, in response to proMMP-9, Mcl-1 sequestered BH3-only proteins thus preventing mitochondrial damage and apoptosis. To confirm this, we studied cytochrome c release by immunofluorescence, using the mitochondrial protein Hsp60 as reference. Figure 2D shows, for a representative sample, that after 24 hr culture on BSA, a lack of colocalization of cytochrome c and Hsp60 was already visible and became very evident after 48 hr, confirming its mitochondrial release. In contrast, colocalization of both proteins was clearly observed on proMMP-9-cultured cells. Accordingly, active Bax, a proapoptotic factor involved in the mitochondrial pathway, was readily detected after 24 hr on BSA-treated cells, but it was mostly absent on proMMP-9-treated cells (Figure 2D). Cytochrome c release was not affected by the caspase inhibitors ZVAD-FMK or Z-IETD-FMK (Figure S2E), indicating that it preceded caspase activation. Hence, caspase-3 activation was not observed in 24 hr cultures (data not shown). However, after 48 hr, caspase-3 was converted to the active 17 kDa form and cleaved its substrate PARP-1 on cells cultured on BSA but not on proMMP-9 (Figure S2F). Caspase-8 activation and degradation of its substrate Bid was also observed on 48 hr BSA cultures, probably due to an apoptosis amplification loop (Garrido et al., 2006). In support of this, the caspase-8 inhibitor Flip was not modulated under BSA or proMMP-9 culture conditions (Figure S2F). Altogether these results indicated that spontaneous apoptosis of B-CLL cells on BSA involved the mitochondrial pathway and initiation of this mechanism was prevented by proMMP-9.

To determine whether Mcl-1 upregulation was required for the proMMP-9 survival effect, we transfected B-CLL cells with two different Mcl-1 siRNAs. Western blotting analyses confirmed that both siRNA efficiently reduced Mcl-1 expression (average 58% and 71% reduction for siRNA<sub>1</sub> and siRNA<sub>2</sub>, respectively), with respect to untransfected or control siRNA-transfected cells (Figure 2E). siRNA transfection did not significantly affect cell adhesion to proMMP-9 (or VCAM-1, used as control) (Figure S2G). *Mcl-1* gene silencing did not reduce the viability of cells on BSA but resulted in complete inhibition of the proMMP-9 anti-apoptotic effect, with viability values falling from 72%–70% (untransfected and control siRNA-transfected cells, respectively) to 52% and 50% for Mcl-1 siRNA<sub>1</sub> and siRNA<sub>2</sub> respectively (Figure 2F). This was accompanied by activation of caspase-3





**Figure 2. Cell Adhesion to proMMP-9 Upregulates Mcl-1 Expression and Prevents Mitochondrial Damage**

(A) A total of  $3 \times 10^6$  B-CLL cells from three different patients were lysed before (Const.) or after 48 hr culture on 0.5% BSA- or 60 nM proMMP-9-coated plates and analyzed by western blotting. Values represent the average Bcl-2 family protein/actin ratio after normalizing constitutive values to 1.

(B)  $10^7$  B-CLL cells were cultured on BSA or proMMP-9 for 2 hr and Mcl-1 mRNA expression was analyzed by RT-PCR. Normalized average values are shown.

(C) Lysates from  $2 \times 10^7$  B-CLL cells cultured for 48 hr on BSA or proMMP-9 were immunoprecipitated with anti-Mcl-1 Abs or rabbit IgG (Ctl) and analyzed by western blotting.

(D) B-CLL cells cultured on BSA or proMMP-9 for 24 or 48 hr were analyzed by confocal microscopy using Abs for cytochrome c or active Bax, followed by Alexa488-labeled secondary Abs. Hsp60 was detected with specific Abs and Texas red-labeled secondary Abs. Colocalization of cytochrome c and Hsp60 was further demonstrated by dot-plot analyses. Scale bar, 10  $\mu$ m.

(E) B-CLL cells were transfected or not with the indicated siRNAs and analyzed by western blotting. Representative blots from four samples are shown. Numbers represent the average (six samples) Mcl-1/actin ratio after normalizing untransfected cell values to 1.

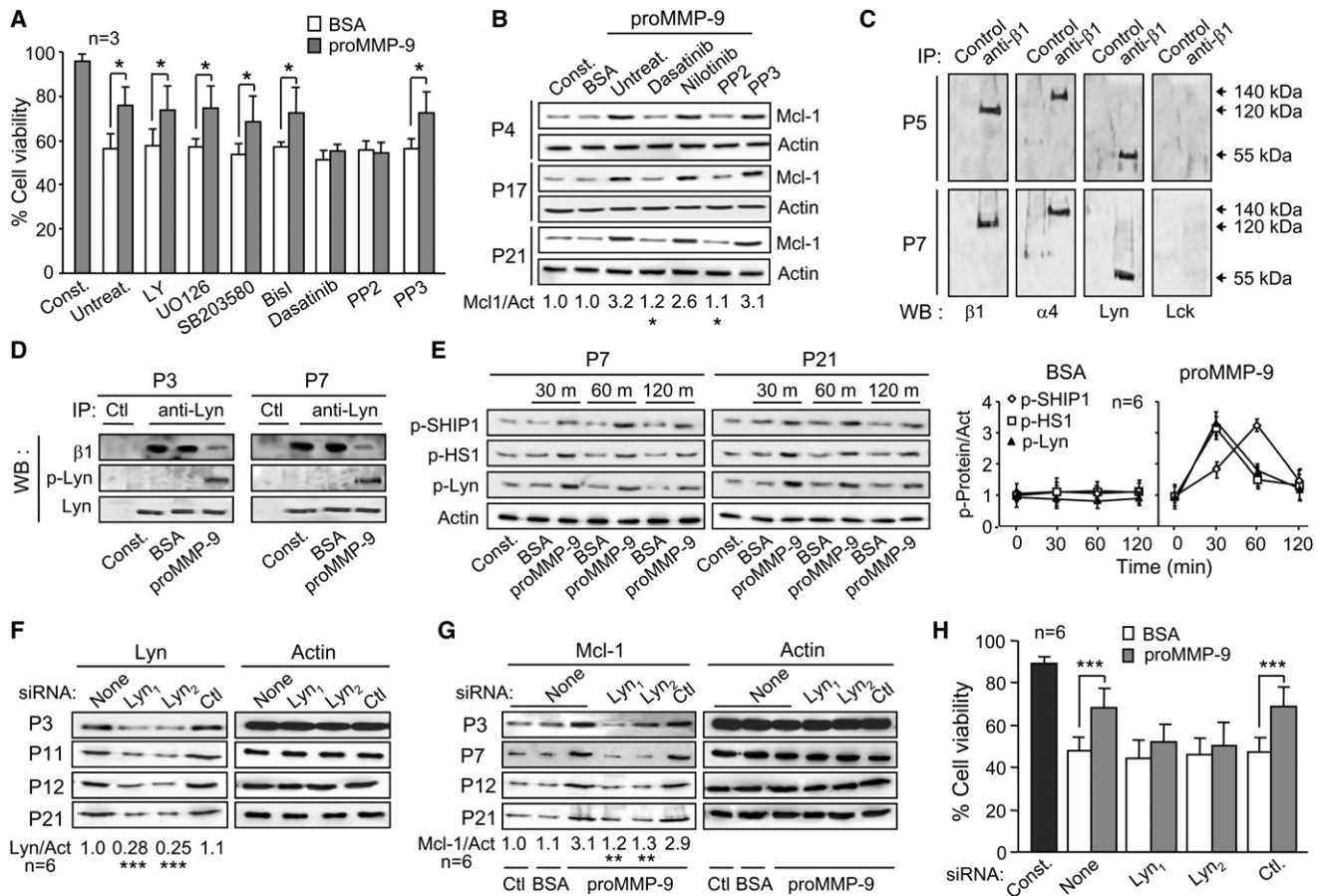
(F) The same samples shown in (E) were cultured on BSA or proMMP-9 and viability was determined after 48 hr by flow cytometry. Average values  $\pm$  SD are shown. \*\*\* $p \leq 0.001$ . See also Figure S2.

and cleavage of PARP-1 (Figure S2H). These results established that Mcl-1 was a crucial component of the proMMP-9 survival effect.

### The Src Family Kinase Lyn Is Responsible for the proMMP-9 Antiapoptotic Function

To determine the signaling pathway involved in the proMMP-9 survival function, we first cultured B-CLL cells on BSA or proMMP-9 in the absence or presence of specific kinase inhibitors and measured their viability after 48 hr. Figure 3A shows that blocking PI3K/Akt, MEK, p38 MAPK, or PKC signaling did not prevent the antiapoptotic activity of proMMP-9. However, blocking Src family kinase (SFK) signaling with PP2 or dasatinib

reduced the viability of cells on proMMP-9 to the levels of cells on BSA; PP3, an inactive analog of PP2, had no effect. Consequently, in the presence of PP2 or dasatinib, but not of PP3 or the B cell receptor (BCR) inhibitor nilotinib, Mcl-1 was not upregulated by proMMP-9 (Figure 3B) and cells were no longer protected against caspase activation (Figure S3A). We next aimed to identify the SFK responsible for the observed survival signaling. B-CLL cells mainly express the Lyn and Lck members of this family (Majolini et al., 1998) and we confirmed the presence of these two kinases in lysates of two representative B-CLL samples (Figure S3B). As signaling was induced upon proMMP-9 binding to  $\alpha\beta 1$  integrin and CD44v (Figure 1D), we next studied whether Lyn or Lck constitutively interacted with these receptors. Lysates from fresh B-CLL cells were immunoprecipitated with anti- $\beta 1$  or anti-CD44 Abs and analyzed by western blotting. Figure 3C shows that anti- $\beta 1$  Abs pulled down the  $\beta 1$  and  $\alpha 4$  integrin subunits as expected, plus one band of 55 kDa, which was recognized by anti-Lyn but not by anti-Lck Abs. Lyn was absent in the anti-CD44 immunoprecipitates (data not shown), indicating the specific association of this kinase with  $\alpha\beta 1$  integrin. Conversely, western



**Figure 3. Lyn Kinase Signaling Is Involved in proMMP-9-Induced Cell Survival and Mcl-1 Upregulation**

(A) B-CLL cells were incubated on BSA- or proMMP-9-coated wells in the presence or absence of the indicated inhibitors and viability was measured after 48 hr by flow cytometry.

(B) B-CLL cells treated or not with the indicated inhibitors were added to BSA- or proMMP-9-coated wells. After 48 hr cells were lysed and Mcl-1 was analyzed by western blotting. Numbers represent normalized average Mcl-1/actin values.

(C) Lysates from fresh B-CLL cells were immunoprecipitated with anti- $\beta$ 1 integrin subunit mAbs and analyzed by western blotting.

(D) B-CLL cells were lysed before or after 30 min culture on BSA- or proMMP-9-coated plates. Lysates were immunoprecipitated with anti-Lyn pAbs and analyzed by western blotting.

(E) A total of  $3 \times 10^6$  B-CLL cells, cultured on BSA or proMMP-9 for the indicated times, were lysed and protein phosphorylation was analyzed by western blotting. Representative blots for two patient samples and average values for six samples are shown.

(F) A total of  $2 \times 10^6$  B-CLL cells were transfected or not with the indicated siRNAs and the efficiency of the transfection was analyzed by western blotting. Normalized average values (n = 6) are shown.

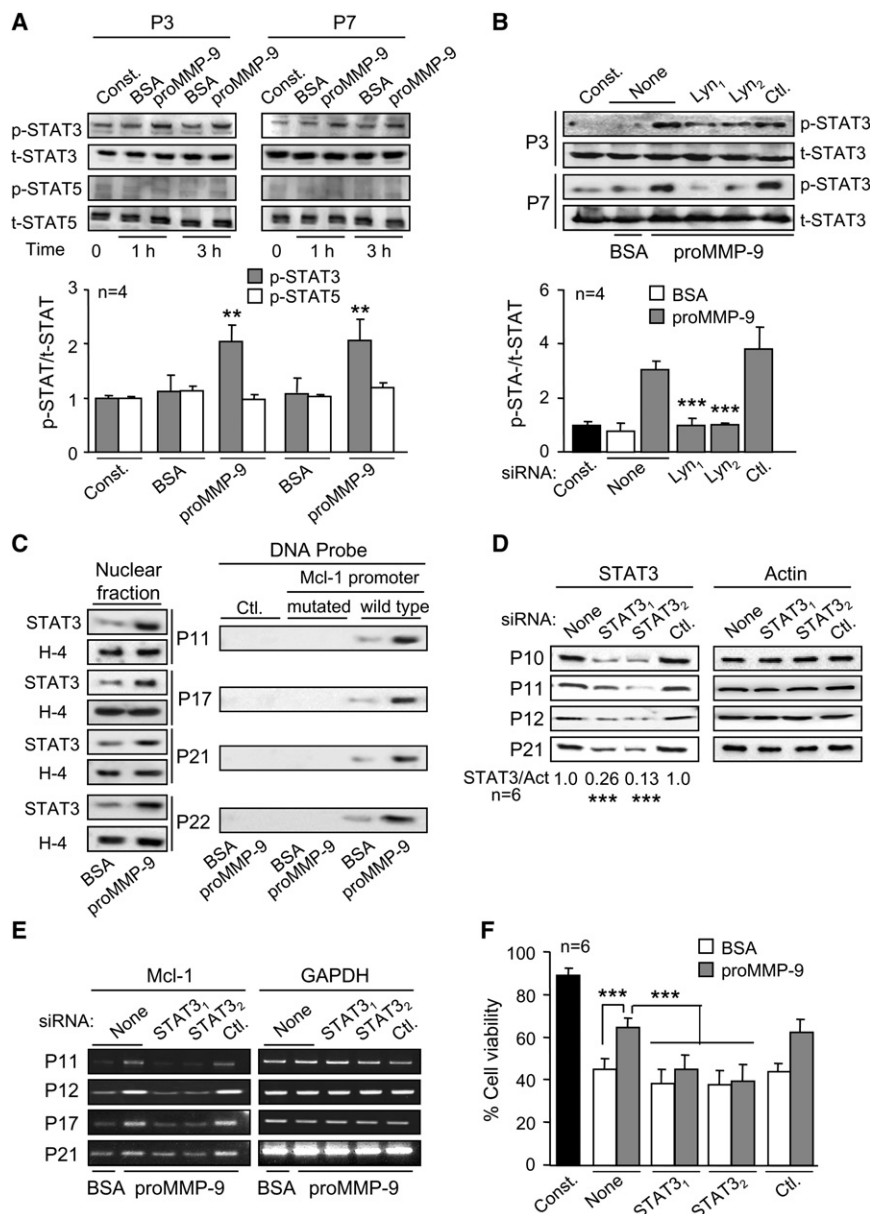
(G) The same cell samples used in (F) were cultured on BSA or proMMP-9 for 48 hr, lysed, and analyzed for Mcl-1 expression by western blotting. Normalized average values  $\pm$  SD are shown.

(H) An aliquot of the cells used in (G) was removed before lysis and viability was determined by flow cytometry. \* $p \leq 0.05$ , \*\*\* $p \leq 0.001$ . See also Figure S3.

blotting analyses of anti-Lyn immunoprecipitates revealed the presence of the  $\alpha$ 4 and  $\beta$ 1 integrin subunits (Figure S3C). These results were further confirmed by immunofluorescence analyses using confocal microscopy. Figure S3D shows that for a representative sample the  $\alpha$ 4/ $\beta$ 1 subunits, but not CD44, colocalized with Lyn at the cell periphery, as documented by dot-plot analysis.

The preceding experiments clearly established that Lyn was bound to  $\alpha$ 4/ $\beta$ 1 integrin in unstimulated B-CLL cells. We next studied whether  $\alpha$ 4/ $\beta$ 1/proMMP-9 interaction affected this binding and/or activated Lyn. Lysates of cells that had been incubated on BSA or proMMP-9 for 30 min were immunoprecipitated

with anti-Lyn Abs and analyzed by western blotting. Figure 3D shows that after cell incubation on BSA, the  $\beta$ 1 integrin subunit remained bound to Lyn, but this association was almost completely lost upon incubation on proMMP-9. To determine whether this was due to Lyn activation, we incubated the same lysates with anti-p-Lyn Tyr396 Ab and analyzed them by western blotting. Indeed, cell binding to proMMP-9 clearly resulted in Lyn Tyr396 phosphorylation while incubation on BSA did not (Figure 3D). In agreement with this, the Lyn substrates HS1 and SHIP1 were phosphorylated on proMMP-9-cultured cells in correlation with Lyn activation (Figure 3E). Both substrates as well as Lyn were already phosphorylated after 15 min (data



**Figure 4. Cell Adhesion to proMMP-9 Induces STAT3 Phosphorylation and Binding to the Mcl-1 Promoter in a Lyn-Dependent Manner**

(A) B-CLL cells were lysed before (Const.) or after culture on BSA- or proMMP-9-coated plates. STAT3 and STAT5 phosphorylation was analyzed by western blotting and average values ( $n = 4$ ) are shown.

(B) B-CLL cells transfected or not with Lyn or control siRNAs were cultured on BSA or proMMP-9 for 1 hr, lysed, and analyzed by western blotting.

(C) B-CLL cells were cultured on BSA or proMMP-9 for 2 hr and the nuclear and cytosolic fractions were separated. Nuclear extracts were incubated with the indicated biotinylated DNA probes and streptavidin particles and analyzed by western blotting. The H4 histone was used as an internal nuclear marker.

(D) B-CLL cells were transfected or not with the indicated siRNAs and, after 24 hr, cells were lysed and analyzed by western blotting.

(E) Individual B-CLL cells from four patients transfected or not with STAT3 or control siRNA were cultured on BSA or proMMP-9 for 2 hr. Cells were collected and Mcl-1 mRNA expression was analyzed by RT-PCR using GAPDH as internal control.

(F) The viability of untransfected and siRNA-transfected B-CLL cells after 48 hr culture on BSA or proMMP-9 was determined by flow cytometry. Average values  $\pm$  SD are shown. \* $p \leq 0.05$ , \*\*\* $p \leq 0.001$ . See also Figure S4.

not shown), reaching a maximum after 30 min (HS1 and Lyn) or 60 min (SHIP1) of proMMP-9 exposure (Figure 3E). As observed for Mcl-1 (Figure 3B), the BCR inhibitors imatinib and nilotinib did not affect proMMP-9-induced Lyn phosphorylation, although this was abolished by PP2 or dasatinib (Figure S3E). Likewise, the BCR inhibitors (or a Janus kinase-2 inhibitor; data not shown) did not affect the increased viability of proMMP-9-cultured cells at any of the concentrations tested (Figure S3F).

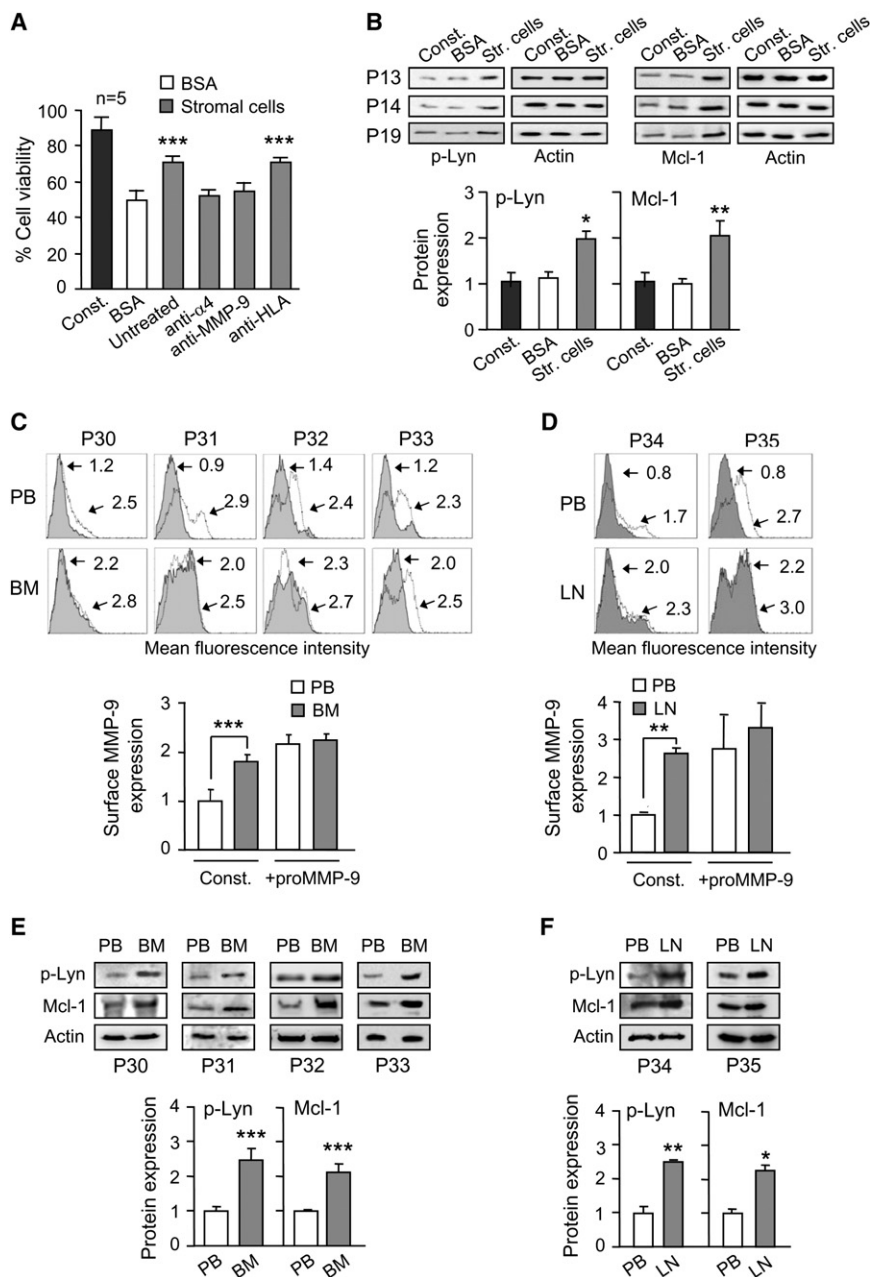
Having established that Lyn was specifically activated by proMMP-9, we studied whether Lyn was necessary for the observed survival effect. B-CLL cells ( $n = 6$ ) were transfected with two different Lyn siRNAs and analyzed for Mcl-1 expression and cell viability. Figure 3F shows that both siRNAs significantly reduced Lyn expression (average 72% and 75%, respectively), compared to untransfected or control siRNA-transfected cells.

clearly established that Lyn plays a key role in the anti-apoptotic signaling induced by cell adhesion to proMMP-9.

#### B-CLL Cell Adhesion to proMMP-9 Induces STAT3 Phosphorylation in a Lyn-Dependent Manner

To further explore the signaling pathway initiated by proMMP-9 and leading to Lyn activation and Mcl-1 upregulation, we studied the transcription factors involved. SFK have been shown to phosphorylate STAT factors (Silva, 2004; Hayakawa and Naoe, 2006), and STAT3 and STAT5 can regulate Mcl-1 expression (Akagi, 2009). We therefore analyzed whether STAT3 and STAT5 were phosphorylated upon cell adhesion to proMMP-9. STAT3 phosphorylation was appreciable after 30 min of adhesion (data not shown) and was maximal (2.1-fold increase,  $p \leq 0.01$ ) after 1 hr (Figure 4A), whereas STAT5 was not





**Figure 5. B-CLL Cells Cultured on Primary Stromal Cells or Isolated from Lymphoid Tissues Show Increased Surface-Bound MMP-9, p-Lyn, and Mcl-1 and Viability**

(A) B-CLL cells from five patients were individually cultured on BSA or primary stromal cells for 48 hr in the presence or absence of the indicated Abs. Cell viability was determined by flow cytometry and average values are shown.

(B) B-CLL cells from three of the samples used in (A) were lysed after 30 min (p-Lyn) or 48 hr (Mcl-1) culture on BSA or stromal cells (Str.) and analyzed by western blotting. Normalized average values are shown.

(C and D) Fresh B-CLL cells from the BM and PB (n = 4) (C) or LN and PB (n = 2) (D) of the same patient, were incubated (30 min, 4°C) with or without 60 nM of soluble proMMP-9. Cells were treated with an anti-MMP-9 Ab and secondary Abs and analyzed by flow cytometry. Shaded areas represent constitutive expression and dotted lines indicate exogenous proMMP-9 bound. Average values after normalizing PB constitutive expression to 1 are shown.

(E and F) B-CLL cells isolated from BM and PB (E) or LN and PB (F) were lysed and analyzed by western blotting. pLyn/actin and Mcl-1/actin average values were obtained after normalizing PB values to 1. Error bars represent SD. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001. See also Figure S5.

phosphorylated (Figure 4A). Thus STAT3 and SHIP1 phosphorylation followed similar kinetics (Figures 3E and 4A). STAT3 phosphorylation persisted after 3 hr (Figure 4A) and was no longer visible after 6 hr (data not shown). The Lyn kinase was required for STAT3 activation, as it was abolished by *Lyn* gene silencing (Figure 4B). Next, to establish that STAT3 activation was responsible for the increased *Mcl-1* mRNA transcription (Figure 2B), we analyzed whether STAT3 bound to the *Mcl-1* promoter. Nuclear extracts from B-CLL cells that had been cultured for 2 hr on BSA or proMMP-9 were incubated with biotinylated DNA probe and streptavidin-labeled beads and analyzed by western blotting. STAT3 was clearly increased in the nuclear fraction of proMMP-9-treated cells compared to BSA-treated cells (Figure 4C). Moreover, STAT3 from

STAT3 activation that results in *Mcl-1* upregulation and cell survival.

#### B-CLL Cells Present in Lymphoid Organs Display Higher Surface MMP-9 Expression, Lyn Activation, and Mcl-1 Levels than Their Peripheral Blood Counterparts

We next studied whether the outlined survival pathway was observed in the pathophysiological context of B-CLL. As a first approach, B-CLL cells were co-cultured with primary stromal cells derived from a B-CLL patient, and the viability was measured after 48 hr. Under these conditions B-CLL cells had significantly higher viability than BSA-cultured cells (Figure 5A), confirming previous reports (Lagneaux et al., 1998; Ringshausen et al., 2004). The survival effect was inhibited by anti- $\alpha 4$  integrin subunit



and anti-MMP-9 Abs (Figure 5A), confirming the involvement of both molecules. Importantly, B-CLL cell surface MMP-9 expression increased >2-fold upon stromal cell contact (Figure S5A). Furthermore, analyses of lysates of B-CLL cells that had been in contact with stromal cells revealed significant upregulation of p-Lyn and Mcl-1, compared to BSA cell cultures (Figure 5B).

We then reasoned that B-CLL cells, when present in lymphoid organs, upregulate their surface MMP-9 expression (by binding endogenous and/or exogenous MMP-9) and activate the survival pathway. To address this, B-CLL cells were isolated from the PB and BM ( $n = 4$ ) or PB and lymph nodes (LN;  $n = 2$ ) of individual patients and their MMP-9 expression was analyzed by flow cytometry. Figures 5C and 5D show that BM and LN B-CLL cells constitutively expressed more surface MMP-9 (average 1.8-fold,  $p \leq 0.001$ , and 2.6-fold,  $p \leq 0.01$ , for BM and LN, respectively) than their PB counterparts. Accordingly, PB cells were able to bind exogenously added proMMP-9, increasing its surface expression by >2-fold. BM or LN cells, however, only raised this expression by 1.2-fold (Figures 5C and 5D). In agreement with an elevated MMP-9 expression, BM and LN cells had significantly higher constitutive levels of p-Lyn and Mcl-1 than PB cells (Figures 5E and 5F). Consequently, and although the constitutive viability of PB and BM (or LN) cells was similar, after 48 hr culture BM cells showed increased viability compared to PB cells (65% versus 41%,  $p \leq 0.05$ ) (Figure S5B). These results clearly established that B-CLL cells increase their surface MMP-9 in lymphoid tissues and that the outlined proMMP-9 survival pathway is active in B-CLL.

#### Comparison between the Survival Pathways Triggered by proMMP-9 and VCAM-1 Reveals Different Signaling by Two $\alpha 4 \beta 1$ Integrin Ligands

We and others previously showed that cell adhesion to VCAM-1 via  $\alpha 4 \beta 1$  integrin prevents spontaneous apoptosis of B-CLL cells (Lagneaux et al., 1998; De la Fuente et al., 1999). We therefore studied if VCAM-1 induced the same survival pathway as proMMP-9. B-CLL cells were incubated on VCAM-1 in the presence or absence of PI3-K, SFK, or ERK1/2 inhibitors, and cell viability was determined after 48 hr. In the absence of inhibitors, VCAM-1 had a similar anti-apoptotic effect (68% average viability) (Figure 6A) as proMMP-9 (Figures 1C–1E). At difference with the proMMP-9 induced signaling, blocking PI3-K with LY294002 reduced the viability of cells attached to VCAM-1 to 48.2%, while the SFK inhibitors dasatinib and PP2 did not (Figure 6A). The inactive analog PP3 or the ERK1/2 inhibitor UO126 had no effect (Figure 6A). In agreement with these results, adhesion of B-CLL cells to VCAM-1 resulted in phosphorylation of Akt but not of Lyn, while adhesion to proMMP-9 induced Lyn phosphorylation as expected, without activating Akt (Figure 6B).

We next studied whether cell adhesion to VCAM-1 upregulated Mcl-1. Figure 6C shows that Mcl-1 expression was not upregulated by cell adhesion to VCAM-1 and, consequently, Mcl-1 gene silencing did not affect the VCAM-1-induced anti-apoptotic effect (Figure 6D). In contrast, Bcl-xL, which was not modulated upon adhesion to proMMP-9 (Figure 2A), was significantly induced (1.8-fold) by VCAM-1 (Figure 6C).

To determine whether both survival pathways cooperated, we incubated B-CLL cells on proMMP-9, VCAM-1, or both and measured the viability after 48 hr. Figure 6E shows that cells

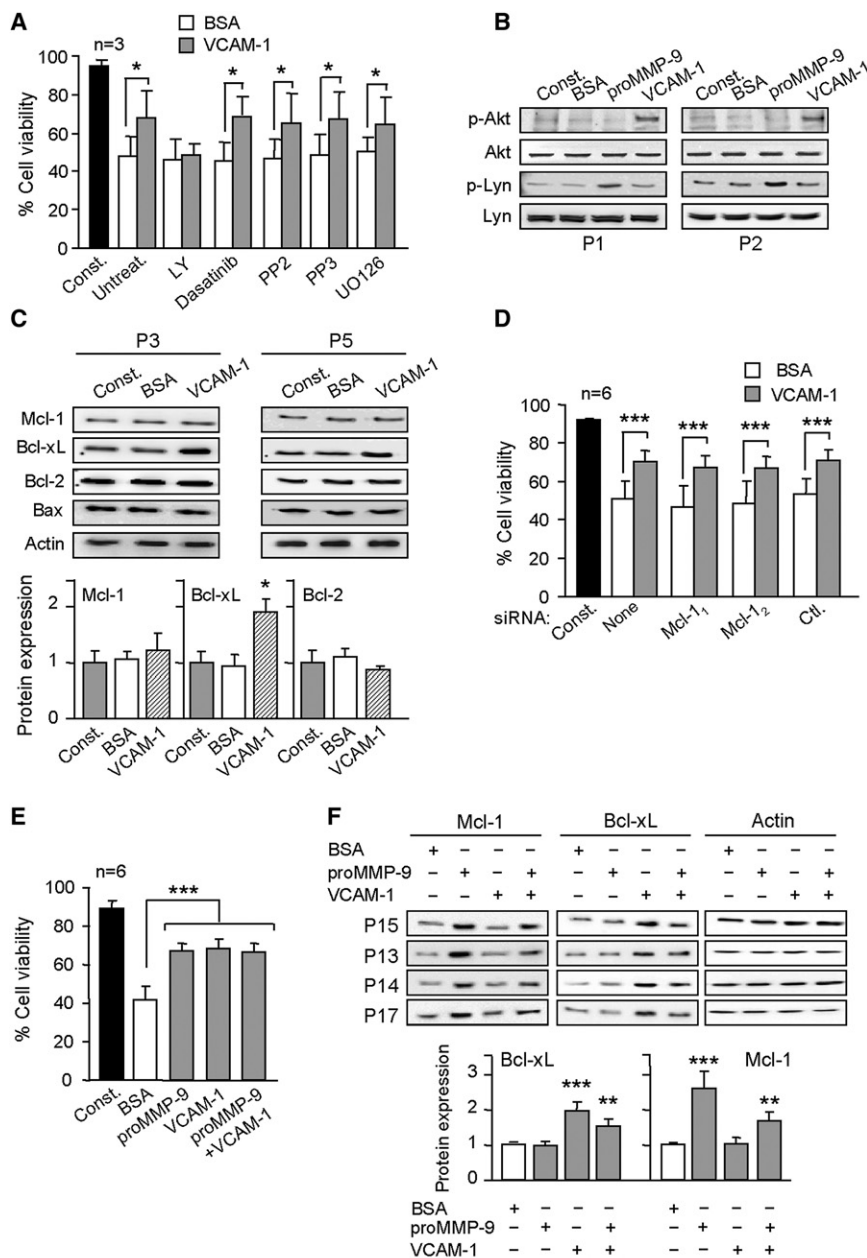
cultured in mixed substrata had similar viability than when cultured on proMMP-9 or VCAM-1 alone, ruling out a cooperative effect. Upregulation of Bcl-xL and Mcl-1 on mixed substrata was significant, but was lower than when triggered by each protein alone (Figure 6F), suggesting a possible competition between both pathways. Altogether these results established that the survival pathway induced by proMMP-9 was clearly different from that elicited by VCAM-1, albeit both proteins are ligands for  $\alpha 4 \beta 1$  integrin. A schematic representation of both pathways is shown in Figure 7.

#### DISCUSSION

We have studied the functional consequences of proMMP-9 binding to its receptors,  $\alpha 4 \beta 1$  integrin and CD44v, in B-CLL cells. We report that proMMP-9, either immobilized or in soluble form, induced an intracellular signaling pathway leading to cell survival. An anti-apoptotic role for MMP-9 has been previously observed in several experimental models of carcinoma (Meyer et al., 2005; Acuff et al., 2006; Kunigal et al., 2008). MMP-9 also contributed to the survival of B-CLL cells on BM stroma (Ringshausen et al., 2004). In these previous studies, the MMP-9 antiapoptotic effect was due to its enzymatic activity. The present study shows that the sole engagement of proMMP-9 or the hemopexin domains of MMP-9 or MMP-2 to its cell surface receptors induced cell survival signals. This represents a surprising function for an MMP, which does not require its proteolytic activity. Our data also add induction of signal transduction as a new function to the known multifunctional liganding activities of MMP hemopexin domains (Piccard et al., 2007). Whether this property is shared by all hemopexin-containing proteins remains to be established.

The Lyn kinase was a specific and crucial component of the signaling pathway elicited by proMMP-9/receptor interaction. Lyn is constitutively active in B-CLL cells and contributes to their defective apoptosis, as inhibiting this kinase with PP2 (Contri et al., 2005) or dasatinib (Veldurthy et al., 2008), or disruption of an aberrant Lyn/Hsp90 cytosolic complex (Trentin et al., 2008) induced cell death. We show in this report that Lyn was further activated in response to proMMP-9 and this contrasts with the poor Lyn activity increase induced by BCR engagement in B-CLL cells (Contri et al., 2005). Instead, BCR upregulated Mcl-1 and induced cell survival mostly via PI3K/Akt and ERK activation (Petlickovski et al., 2005; Longo et al., 2008). Activation of Lyn by proMMP-9 therefore represents a distinct and BCR-independent mechanism contributing to B-CLL cell survival. Moreover, Lyn was responsible for the subsequent STAT3 phosphorylation and Mcl-1 upregulation. Phosphorylation of STATs by SFK members is an alternative mechanism independent of the canonical JAK-STAT pathway (Silva, 2004; Hayakawa and Naoe, 2006). Nevertheless, the possibility of Lyn acting via an intermediate factor cannot be disregarded. Like in the case of Lyn, both STAT3 and Mcl-1 were crucial and specific components of the proMMP-9-induced survival signaling.

The described antiapoptotic pathway required B-CLL cell solid-phase adhesion or binding in solution to proMMP-9. We have previously shown that  $\alpha 4 \beta 1$  integrin and CD44v are involved in this interaction (Redondo-Muñoz et al., 2008b). Our present



**Figure 6. B-CLL Cell Adhesion to VCAM-1 Induces a Survival Pathway that Is Different from that Induced by proMMP-9**

(A) B-CLL cells from three patients were treated or not with the indicated kinase inhibitors and added to BSA- or 60 nM VCAM-1-coated wells. After 48 hr, cell viability was determined and average values are shown.

(B) B-CLL cells were lysed before or after 30 min culture on BSA, proMMP-9, or VCAM-1 and Lyn and Akt activation was analyzed by western blotting.

(C) B-CLL cells before or after 48 hr culture on BSA- or VCAM-1-coated wells were lysed and Bcl-2 family proteins were analyzed by western blotting. Quantitative values represent the Bcl-2 family protein/actin ratio after normalizing constitutive values to 1.

(D) B-CLL cells from six patients transfected or not with the indicated siRNAs were cultured on BSA or VCAM-1 and viability was measured after 48 hr by flow cytometry.

(E) B-CLL cells from six patients were cultured on the indicated proteins for 48 hr and viability was determined by flow cytometry.

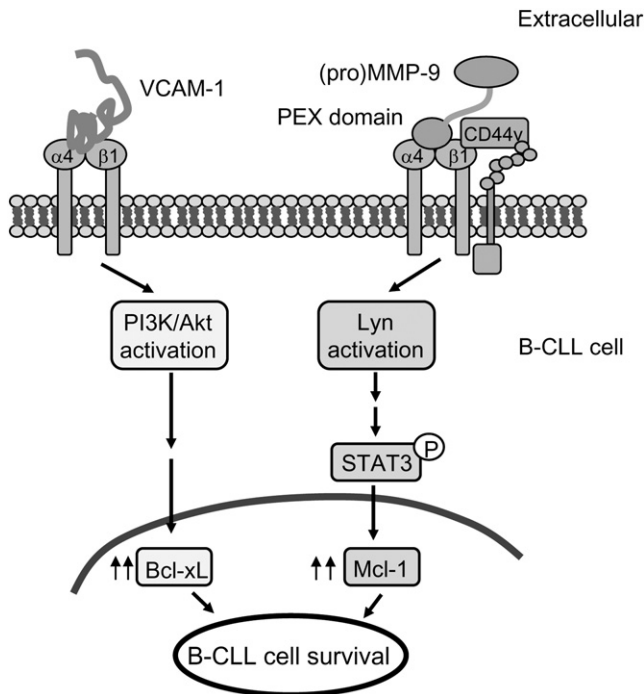
(F) B-CLL cells from four patients were cultured on the indicated proteins for 48 hr, lysed, and analyzed by western blotting. Normalized average values  $\pm$  SD are shown. \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ .

results show that only  $\alpha 4 \beta 1$  associates with Lyn, suggesting that CD44v may stabilize or strengthen this interaction. Contribution of  $\alpha 4 \beta 1$  integrin to B-CLL cell viability and resistance to fludarabine was previously demonstrated upon adhesion to fibronectin, VCAM-1, or BM stromal cells (Lagneaux et al., 1998; De la Fuente et al., 1999, 2002). We now show that induction of cell survival by the  $\alpha 4 \beta 1$ /VCAM-1 axis does not involve the Lyn/STAT3/Mcl-1 pathway, which thus appears to be unique for proMMP-9. This indicates that  $\alpha 4 \beta 1$  integrin may trigger distinct intracellular events depending on the engaged ligand. Accordingly,  $\alpha 4 \beta 1$ -mediated T lymphocyte adhesion to VCAM-1 or fibronectin was shown to differentially upregulate *MMP-2* or *MMP-2/MMP-9* mRNA, respectively (Yakubenko et al., 2000). As our present results further demonstrate that the VCAM-1 and

proMMP-9 survival pathways show a certain degree of competition, the availability (or higher expression) of each ligand at a given location probably determines the signals induced.

The present report therefore establishes that proMMP-9 is a functional ligand for  $\alpha 4 \beta 1$  integrin, able to induce intracellular signals independently of its proteolytic activity. Although B-CLL cells synthesize and bind proMMP-9 (Bauvois et al., 2002; Kamiguti et al., 2004; Redondo-Muñoz et al., 2006, 2008b) our results show that this may not be sufficient to induce the survival pathway, as in the absence of exogenous proMMP-

9, cells become apoptotic after 2 days in culture. Increasing evidence supports the notion that the microenvironment plays an essential role in the proliferation and resistance to apoptosis of B-CLL cells. In vitro experiments have demonstrated that culturing B-CLL cells on several cell types increases B-CLL cell viability (Burger et al., 2000; Lagneaux et al., 1998; Panayiotidis et al., 1996; Pedersen et al., 2002), strongly suggesting that B-CLL cells in niches receive survival signals. In this regard, differential expression of the proapoptotic gene *Noxa* in LN and PB B-CLL cells was recently reported (Smit et al., 2007). We now show that B-CLL cells isolated from BM or LN express significantly higher levels of surface-bound MMP-9 than their PB counterparts, indicating that B-CLL cells acquire more (pro)MMP-9 when present in lymphoid tissues. Both B-CLL cells



**Figure 7. Schematic Representation of the Survival Pathways Induced by MMP-9 and VCAM-1**

$\alpha 4 \beta 1$  integrin mediates survival signals for B-CLL cells by two different pathways. When VCAM-1 binds the integrin, PI3-K is activated and phosphorylates Akt, resulting in upregulation of Bcl-xL. When the hemopexin domain of (pro)MMP-9 (PEX) interacts with the integrin, in conjunction with CD44v, a different signaling cascade is initiated, leading to Lyn activation, STAT3 phosphorylation, and Mcl-1 upregulation. The noncatalytic hemopexin domain is sufficient to induce anti-apoptotic effects in B-CLL cells. This mechanism operates independently from VCAM-1-induced survival, albeit through ligand-binding of  $\alpha 4 \beta 1$  integrin by both molecules.

and tissue resident cells may contribute to this (pro)MMP-9 up-regulation, as further confirmed by our experiments using primary stromal cells. In support of our results, gene microarray analyses have recently shown that *MMP-9* is dramatically upregulated (>25-fold) upon B-CLL cell culture on a murine stromal cell line (Edelmann et al., 2008). Consistent with an elevated *MMP-9* expression on B-CLL cells in tissues, our results also show higher levels of phosphorylated Lyn and Mcl-1 and viability in BM and LN cells than in their PB counterparts. Therefore, the survival pathway that we have identified is active in the physiopathological context of B-CLL. Localization in lymphoid tissues may thus provide a means for B-CLL cells to receive survival stimuli and escape apoptosis, as we show in our study. In these tissues, (pro)MMP-9 may act as an auto-crine or paracrine survival factor for B-CLL and, even in the presence of supra- or equimolar levels of its inhibitor TIMP-1, i.e., without any catalytic activity, the anti-apoptotic effect will persist. Our findings support the recent view that more attention should be paid to the microenvironment as a new therapeutic target in B cell malignancies (Burger et al., 2009) and may also have relevance in solid tumor progression. Increased *MMP-9* and/or *MMP-2* expression correlates with aggressiveness in several neoplasias including melanoma and breast, lung, and

renal carcinomas (Turpeenniemi-Hujanen, 2005). Elevated levels of these MMPs are also present in solid tumor microenvironments and play an important role in angiogenesis and tumor metastasis (Acuff et al., 2006; Deryugina and Quigley, 2006, 2009; Allavena et al., 2008; Bacac and Stamenkovic, 2008). It is therefore possible that part of this (pro)MMP-9 (or (pro)MMP-2) binds to tumor cells (via integrin/hemopexin domain) and induces survival pathways, as we demonstrate for B-CLL. As dual and unexpected roles for MMPs in cancer are now becoming evident (López-Otín and Matrisian, 2007), our results reveal new properties for proMMP-9 and highlight the role of its hemopexin domain in the pathology of B-CLL. Thus, *MMP-9* not only regulates the migration/arrest of B-CLL cells, but also maintains the malignant population by providing survival signals. The anti-apoptotic pathway identified here should help designing future therapies targeting the *MMP-9* hemopexin domain aimed to prevent B-CLL progression.

## EXPERIMENTAL PROCEDURES

### Patients and Cells

Approval was obtained from the review boards of the Hospital Clínico Universitario, Valencia, and the Hospital Universitario Puerta de Hierro, Madrid, for these studies. PB samples from 35 B-CLL patients (Table 1) were obtained after informed consent. BM and LN samples were obtained from four and two of these patients, respectively (Table 1).  $CD5^+$  B-lymphocytes were purified by Ficoll-Hypaque (Nycomed) centrifugation and negative selection with anti-CD3-conjugated Dynabeads (DynaL Biotech ASA). The resulting B cell population was >95%  $CD19^+$  and >90%  $CD5^+$ , determined on a Coulter Epics XL flow cytometer (Beckman Coulter) with the Expo 32 ADC software (Applied Cytometry System). Stromal cells were obtained from the mononuclear cell fraction of one B-CLL patient after 3 week culture in IMDM (GIBCO)/15% FBS, and used for up to 4 weeks.

### Cell Adhesion Assays and Binding Assays in Solution

Adhesion assays were performed on 96-well plates coated with 0.5% BSA, 60 nM proMMP-9 or proMMP-9 variants, or 60 nM VCAM-1.  $2 \times 10^5$  B-CLL cells were incubated with 1.4 ng/ml 2', 7'-bis(carboxyethyl)-5(6')-carboxyfluorescein-acetoxymethyl ester (Molecular Probes) for 20 min, suspended in RPMI 1640/0.5% BSA (adhesion medium), and added to the coated wells. After 90 min at 37°C, attached cells were lysed with PBS/0.1% SDS and quantified using a fluorescence analyzer (BMG Labtechnologies). For binding assays in solution,  $10^5$  cells were incubated in 100  $\mu$ l adhesion medium (for determination of constitutive proMMP-9 expression) or medium containing proMMP-9 or rproMMP-9 variants (all at 60 nM) for 30 min at 4°C. After washing with ice-cold RPMI, cells were incubated with anti-MMP-9 pAbs (30 min, 4°C), washed with cold PBS, and incubated (30 min, 4°C) with Alexa 488-labeled secondary Abs. Surface-bound proMMP-9 was analyzed by flow cytometry.

### Analysis of Apoptosis

A total of  $10^5$  B-CLL cells in 100  $\mu$ l RPMI/0.1% FCS were added to 96-well plates coated with either 0.5% BSA, a primary stromal cell monolayer, or appropriate proteins (all at 60 nM). After 24–48 hr, cells were collected and resuspended in 1 $\times$  binding buffer (Bender Medsystems) containing 3  $\mu$ l of FITC-Annexin V and 50  $\mu$ g of propidium iodide. After 10 min, cells were analyzed by flow cytometry. Cell viability was also analyzed after 48 hr by adding 100  $\mu$ g MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazoliumbromide) (Sigma-Aldrich) and additional incubation for 3 hr in the dark. The blue MTT formazan precipitate was dissolved in 2% SDS (4 hr, room temperature) and the absorbance at 540 nm was determined on a Multiskan Bichromatic microplate reader (Labsystems). For inhibition experiments, cells in adhesion medium were incubated (30 min, 37°C) with appropriate Abs (10  $\mu$ g/ml) or peptides (500  $\mu$ g/ml) or with LY294002 (2.5  $\mu$ M), dasatinib (0.1 and 1  $\mu$ M), imatinib, nilotinib (1 and 5  $\mu$ M each), SB203580, Bis I, UO126, PP2, or PP3 (all at 5  $\mu$ M) prior to seeding into the wells. The effect of soluble proMMP-9



on cell viability was determined upon incubating suspended cells with or without (pro)MMP-9 proteins for 48 hr. The presence of proMMP-9 at the cell surface at this time was confirmed by flow cytometry as described.

#### Streptavidin-Conjugated Beads DNA Pull-down Assay

Binding of STAT3 to the Mcl-1 promoter was assayed by streptavidin-conjugated beads as described previously (Deng et al., 2003, 2006). The following biotinylated 22 nucleotide sequences were used: 5'-CCCTTTTATGGGAA TACTTTTT-3', containing the specific binding site for STAT factors in the Mcl-1 promoter (Moshynska et al., 2004), and 5'-CCCTTAAGTGGGAATA CTTTTT-3', a mutated sequence that precludes binding to the promoter. The 22 nucleotide sequence 5'-AGAGTGGTCACTACCCCTCTG-3', which does not contain any known enhancer element, was included as a negative control. All biotin-labeled double strand DNA probes were synthesized by Sigma-Aldrich.  $3 \times 10^7$  B-CLL cells were cultured on BSA or proMMP-9 for 2 hr and lysed (5 min, 4°C) with buffer A (10 mM HEPES [pH 8.0], 1.5 mM  $MgCl_2$ , 200 mM sucrose, 0.5% Nonidet P-40, 10 mM KCl, 0.5 mM dithiothreitol, and 1 mM EGTA) containing phosphatase and protease inhibitors. The released crude nuclei were collected by microcentrifugation, washed twice with buffer A, and resuspended in buffer B (PBS [pH 7.4], 1.0 mM EDTA, 1.0 mM dithiothreitol, and inhibitors). Nuclei were disrupted by sonication at 4°C, and debris was removed by centrifugation. Nuclear proteins were then mixed with 4  $\mu$ g biotinylated DNA probes and 40  $\mu$ l streptavidin magnetic particles (Roche) for 1 hr at room temperature, with shaking. DNA-bound transactivators were dissociated after washing and analyzed by western blotting.

Additional experimental procedures are available as [Supplemental Information](#).

#### SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and Supplemental Experimental Procedures and can be found with this article online at [doi:10.1016/j.ccr.2009.12.044](https://doi.org/10.1016/j.ccr.2009.12.044).

#### ACKNOWLEDGMENTS

We thank the B-CLL patients who donated samples for this research; Drs. Paloma Sánchez-Mateos and Rafael Samaniego (Hospital Gregorio Marañón, Madrid) for performing the confocal microscopy analyses and for valuable advice; Dr. Patricia Boya, for helpful suggestions; and Ilse Van Aelst for excellent technical assistance. This work was supported by grants PI060400, SAF2009-07035, and RTICC RD06/0020/0011 (to A.G.P.), by grants PI061637 and RTICC RD06/0020/0080 (to M.J.T.) from the Ministerio de Ciencia e Innovación, and by the Fundación de Investigación Médica Mutua Madrileña (to A.G.P.). Research on the human proMMP-9 mutants was supported by the Geconcerteerde OnderzoeksActies (GOA 2007-2011) and the Fund for Scientific Research-Flanders (FWO-Vlaanderen). Research on the murine proMMP-9-PEX domain was supported by grants Ro 957/6-1 and Ro 957/7-1 from the Deutsche Forschungsgemeinschaft. J.R.M. was supported by the Fundación Ramón Areces. P.E.V.D.S. is a postdoctoral fellow of the FWO-Vlaanderen.

Received: March 24, 2009

Revised: August 31, 2009

Accepted: December 2, 2009

Published: February 16, 2010

#### REFERENCES

Acuff, H.B., Carter, K.J., Fingleton, B., Gorden, D.L., and Matrisian, L.M. (2006). MMP9 from bone marrow-derived cells contributes to survival but not to growth of tumor cells in the lung microenvironment. *Cancer Res.* 66, 259–266.

Allavena, P., Sica, A., Solinas, G., Porta, C., and Mantovani, A. (2008). The inflammatory micro-environment in tumor progression: The role of tumor-associated macrophages. *Crit. Rev. Oncol. Hematol.* 66, 1–9.

Alfonso-Pérez, M., López-Giral, S., Quintana, N.E., Loscertales, J., Martín-Jiménez, P., and Muñoz, C. (2006). Anti-CCR7 monoclonal antibodies as a novel tool for the treatment of chronic lymphocytic leukemia. *J. Leukoc. Biol.* 79, 1157–1165.

Akgul, C. (2009). Mcl-1 is a potential therapeutic target in multiple types of cancer. *Cell. Mol. Life Sci.* 66, 1326–1336.

Bacac, M., and Stamenkovic, I. (2008). Metastatic cancer cell. *Annu. Rev. Pathol.* 3, 221–247. [10.1146/annurev.pthmechdis.3.121806.151523](https://doi.org/10.1146/annurev.pthmechdis.3.121806.151523).

Binet, J.L., Auquier, A., Dighiero, G., Chastang, C., Piguier, H., Goasguen, J., Vaugier, G., Potron, G., Colona, P., Oberling, F., et al. (1981). A new prognostic classification of chronic lymphocytic leukemia derived from a multivariate analysis. *Cancer* 48, 198–206.

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

Burger, J.A., Tsukada, N., Burger, M., Zvaifler, N.J., Dell'Aquila, M., and Kipps, T.J. (2000). Blood-derived nurse-like cells protect chronic lymphocytic leukemia B cells from spontaneous apoptosis through stromal cell-derived factor-1. *Blood* 96, 2655–2663.

Burger, J.A., Ghia, P., Rosenwald, A., and Caligaris-Cappio, F. (2009). The microenvironment in mature B-cell malignancies: A target for new treatment strategies. *Blood* 114, 3367–3375.

Caligaris-Cappio, F., and Hamblin, T.J. (2008). B-cell chronic lymphocytic leukemia: A bird of a different feather. *J. Clin. Oncol.* 17, 399–408.

Cauwe, B., Van den Steen, P.E., and Opdenakker, G. (2007). The biochemical, biological, and pathological kaleidoscope of cell surface substrates processed by matrix metalloproteinases. *Crit. Rev. Biochem. Mol. Biol.* 42, 113–185.

Chiorazzi, N., Rai, K.R., and Ferrarini, M. (2005). Chronic lymphocytic leukemia. *N. Engl. J. Med.* 352, 804–815.

Contri, A., Brunati, A.M., Trentin, L., Cabrelle, A., Miorin, M., Cesaro, L., Pinna, L.A., Zambello, R., Semenzato, G., and Donella-Deana, A. (2005). Chronic lymphocytic leukemia B cells contain anomalous Lyn tyrosine kinase, a putative contribution to defective apoptosis. *J. Clin. Invest.* 115, 369–378.

De la Fuente, M.T., Casanova, B., García-Gila, M., Silva, A., and García-Pardo, A. (1999). Fibronectin interaction with  $\alpha 4 \beta 1$  integrin prevents apoptosis in B-cell chronic lymphocytic leukemia: Correlation with Bcl-2 and Bax. *Leukemia* 13, 266–274.

De la Fuente, M.T., Casanova, B., Moyano, J.V., García-Gila, M., Sanz, L., García-Marco, J., Silva, A., and García-Pardo, A. (2002). Engagement of  $\alpha 4 \beta 1$  integrin (CD49d/CD29) by fibronectin induces in vitro resistance of B chronic lymphocytic leukemia cells to fludarabine. *J. Leukoc. Biol.* 71, 495–502.

Deng, W.G., Zhu, Y., Montero, A., and Wu, K.K. (2003). Quantitative analysis of binding of transcription factor complex to biotinylated DNA probe by a streptavidin-agarose pulldown assay. *Anal. Biochem.* 323, 12–18.

Deng, W.G., Tang, S.T., Tseng, H.P., and Wu, K.K. (2006). Melatonin suppresses macrophage cyclooxygenase-2 and inducible nitric oxide synthase expression by inhibiting p52 acetylation and binding. *Blood* 108, 518–524.

Deryugina, E.I., and Quigley, J.P. (2006). Matrix metalloproteinases and tumor metastasis. *Cancer Metastasis Rev.* 25, 9–34.

Deryugina, E.I., and Quigley, J.P. (2009). Pleiotropic roles of matrix metalloproteinases in tumor angiogenesis: Contrasting, overlapping and compensatory functions. *Biochim. Biophys. Acta*, in press. Published online October 2, 2009. [10.1016/j.bbamer.2009.09.017](https://doi.org/10.1016/j.bbamer.2009.09.017).

Edelmann, J., Klein-Hitpass, L., Carpinteiro, A., Führer, A., Sellmann, L., Stülgensbauer, S., Dührsen, U., and Dürig, J. (2008). Bone marrow fibroblasts induce expression of PI3K/NF- $\kappa$ B pathway genes and a pro-angiogenic phenotype in CLL cells. *Leuk. Res.* 32, 1565–1572.

Egeblad, M., and Werb, Z. (2002). New functions for the matrix metalloproteinases in cancer progression. *Nat. Rev. Cancer* 2, 161–176.

Fridman, R., Toth, M., Chvyrkova, I., Meroueh, S.O., and Mobashery, S. (2003). Cell surface association of matrix metalloproteinase-9 (gelatinase B). *Cancer Metastasis Rev.* 22, 153–166.



- Garrido, C., Galluzzi, L., Brunet, M., Puig, P.E., Didelot, C., and Kroemer, G. (2006). Mechanisms of cytochrome c release from mitochondria. *Cell Death Differ.* 13, 1423–1433.
- Hayakawa, F., and Naoe, T. (2006). SFK-STAT pathway: An alternative and important way to malignancies. *Ann. N Y Acad. Sci.* 1086, 213–222.
- Hu, J., Van den Steen, P.E., Sang, Q.X., and Opdenakker, G. (2007). Matrix metalloproteinase inhibitors as therapy for inflammatory and vascular diseases. *Nat. Rev. Drug Discov.* 6, 480–498.
- Kamiguti, A.S., Lee, E.S., Till, K.J., Harris, R.J., Glenn, M.A., Lin, K., Chen, H.J., Zuzel, M., and Cawley, J.C. (2004). The role of matrix metalloproteinase-9 in the pathogenesis of chronic lymphocytic leukemia. *Br. J. Haematol.* 125, 128–140.
- Kunigal, S., Lakka, S.S., Joseph, P., Estes, N., and Rao, J.S. (2008). Matrix metalloproteinase-9 inhibition down-regulates radiation-induced nuclear factor- $\kappa$ B activity leading to apoptosis in breast tumors. *Clin. Cancer Res.* 14, 3617–3626.
- Lagneaux, L., Delforge, A., Bron, D., De Bruyn, C., and Stryckmans, P. (1998). Chronic lymphocytic leukemic B cells but not normal B cells are rescued from apoptosis by contact with normal bone marrow stromal cells. *Blood* 91, 2387–2396.
- Longo, P.G., Laurenti, L., Gobessi, S., Sica, S., Leone, G., and Efremov, D.G. (2008). The Akt/Mcl-1 pathway plays a prominent role in mediating antiapoptotic signals downstream of the B-cell receptor in chronic lymphocytic leukemia B cells. *Blood* 111, 846–855.
- López-Otin, C., and Matrisian, L.M. (2007). Emerging roles of proteases in tumor suppression. *Nat. Rev. Cancer* 7, 800–808.
- Majolini, M.B., D'Elios, M.M., Galieni, P., Boncristiano, M., Lauria, F., Del Prete, G., Telford, J.L., and Baldari, C.T. (1998). Expression of the T-cell-specific tyrosine kinase Lck in normal B-1 cells and in chronic lymphocytic leukemia B cells. *Blood* 91, 3390–3396.
- Meyer, E., Vollmer, J.Y., Bovey, R., and Stamenkovic, I. (2005). Matrix metalloproteinases 9 and 10 inhibit protein kinase C-potentiates, p53-mediated apoptosis. *Cancer Res.* 65, 4261–4272.
- Moshynska, O., Sankaran, K., Pahwa, P., and Saxena, A. (2004). Prognostic significance of a short sequence insertion in the MCL-1 promoter in chronic lymphocytic leukemia. *J. Natl. Cancer Inst.* 96, 673–682.
- Opdenakker, G., Van den Steen, P.E., and Van Damme, J. (2001). Gelatinase B: A tuner and amplifier of immune functions. *Trends Immunol.* 22, 571–579.
- Panayiotidis, P., Jones, D., Ganeshaguru, K., Foroni, L., and Hoffbrand, A.V. (1996). Human bone marrow stromal cells prevent apoptosis and support the survival of chronic lymphocytic leukaemia cells in vitro. *Br. J. Haematol.* 92, 97–103.
- Pedersen, I.M., Kitada, S., Leoni, L.M., Zapata, J.M., Karras, J.G., Tsukada, N., Kipps, T.J., Choi, Y.S., Bennett, F., and Reed, J.C. (2002). Protection of B-CLL cells by a follicular dendritic cell line is dependent on induction of Mcl-1. *Blood* 100, 1795–1801.
- Pettickovski, A., Laurenti, L., Li, X., Marietti, S., Chiusolo, P., Sica, S., Leone, G., and Efremov, D.G. (2005). Sustained signaling through the B-cell receptor induces Mcl-1 and promotes survival of chronic lymphocytic leukemia B cells. *Blood* 105, 4820–4827.
- Piccard, H., Van den Steen, P.E., and Opdenakker, G. (2007). Hemopexin domains as multifunctional ligand modules in matrix metalloproteinases and other proteins. *J. Leukoc. Biol.* 81, 870–892.
- Rai, K.R., Sawitsky, A., Cronkite, E.P., Chanana, A.D., Levy, R.N., and Paster-nack, B.S. (1975). Clinical staging of chronic lymphocytic leukemia. *Blood* 46, 219–234.
- Redondo-Muñoz, J., Escobar-Díaz, E., Samaniego, R., Perol, M.J., García-Marco, J.A., and García-Pardo, A. (2006). MMP-9 in B-cell chronic lymphocytic leukemia is up-regulated by  $\alpha$ 4 $\beta$ 1 integrin or CXCR4 engagement via distinct signaling pathways, localizes to podosomes, and is involved in cell invasion and migration. *Blood* 108, 3143–3151.
- Redondo-Muñoz, J., Terol, M.J., García-Marco, J.A., and García-Pardo, A. (2008a). MMP-9 is upregulated by CCL21/CCR7 interaction via ERK1/2 signaling and is involved in CCL21-driven B-CLL cell invasion and migration. *Blood* 111, 383–386.
- Redondo-Muñoz, J., Ugarte-Berzal, E., García-Marco, J.A., del Cerro, M.H., Van den Steen, P.E., Opdenakker, G., Terol, M.J., and García-Pardo, A. (2008b).  $\alpha$ 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, 169–178.
- Ringshausen, I., Dechow, T., Schneller, F., Weick, K., Oelsner, M., Peschel, C., and Decker, T. (2004). Constitutive activation of the MAPkinase p38 is critical for MMP-9 production and survival of B-CLL cells on bone marrow stromal cells. *Leukemia* 18, 1964–1970.
- Roeb, E., Schleinkofer, K., Kernebeck, T., Pötsch, S., Jansen, B., Behrmann, I., Matern, S., and Grötzinger, J. (2002). The matrix metalloproteinase 9 (MMP-9) hemopexin domain is a novel gelatin binding domain and acts as an antagonist. *J. Biol. Chem.* 277, 50326–50332.
- Silva, C.M. (2004). Role of STATs as downstream signal transducers in Src family kinase-mediated tumorigenesis. *Oncogene* 23, 8017–8023.
- Smit, L.A., Hallaert, D.Y.H., Spijker, R., de Goeij, B., Jaspers, A., Kater, A.P., Van Oers, M.H.J., van Noessel, C.J.M., and Eldering, E. (2007). Differential Noxa/Mcl-1 balance in peripheral versus lymph node chronic lymphocytic leukemia cells correlates with survival capacity. *Blood* 109, 1660–1668.
- Stamenkovic, I. (2003). Extracellular matrix remodelling: The role of matrix metalloproteinases. *J. Pathol.* 200, 448–464.
- Till, K.J., Lin, K., Zuzel, M., and Cawley, J.C. (2002). The chemokine receptor CCR7 and  $\alpha$ 4 integrin are important for migration of chronic lymphocytic leukemia cells into lymph nodes. *Blood* 99, 2977–2984.
- Till, K.J., Spiller, D.G., Harris, R.J., Chen, H., Zuzel, M., and Cawley, J.C. (2005). CLL, but not normal, B cells are dependent on autocrine VEGF and  $\alpha$ 4 $\beta$ 1 integrin for chemokine-induced motility on and through endothelium. *Blood* 105, 4813–4819.
- Trentin, L., Frasson, M., Donella-Deana, A., Frezzato, F., Pagano, M.A., Tibaldi, E., Gatazo, C., Zambello, R., Semenzato, G., and Brunati, A.M. (2008). Geldanamycin-induced Lyn dissociation from aberrant Hsp90-stabilized cytosolic complex is an early event in apoptotic mechanisms in B-chronic lymphocytic leukemia. *Blood* 112, 4665–4674.
- Turpeenniemi-Hujanen, T. (2005). Gelatinases (MMP-2 and -9) and their natural inhibitors as prognostic indicators in solid cancers. *Biochimie* 87, 287–297.
- Van den Steen, P.E., Dubois, B., Nelissen, I., Rudd, P.M., Dwek, R.A., and Opdenakker, G. (2002). Biochemistry and molecular biology of gelatinase B or matrix metalloproteinase-9 (MMP-9). *Crit. Rev. Biochem. Mol. Biol.* 37, 375–536.
- Van den Steen, P.E., Van Aelst, I., Hvidberg, V., Piccard, H., Fiten, P., Jacobsen, C., Moestrup, S.K., Fry, S., Royle, L., Wormald, M.R., et al. (2006). The hemopexin and O-glycosylated domains tune gelatinase B/MMP-9 bioavailability via inhibition and binding to cargo receptors. *J. Biol. Chem.* 281, 18626–18637.
- Veldurthy, A., Patz, M., Hagist, S., Pallasch, C.P., Wendtner, C.M., Hallek, M., and Krause, G. (2008). The kinase inhibitor dasatinib induces apoptosis in chronic lymphocytic leukemia cells in vitro with preference for a subgroup of patients with unmutated IgV<sub>H</sub> genes. *Blood* 112, 1443–1452.
- Xu, D., Suenaga, N., Edelmann, M.J., Fridman, R., Muschel, R.J., and Kessler, B.M. (2008). Novel MMP-9 substrates in cancer cells revealed by a label-free quantitative proteomics approach. *Mol. Cell. Proteomics* 7, 2215–2228.
- Yakubenko, V.P., Lobb, R.R., Plow, E.F., and Ugarova, T.P. (2000). Differential induction of gelatinase B (MMP-9) and gelatinase A (MMP-2) in T lymphocytes upon  $\alpha$ (4) $\beta$ (1)-mediated adhesion to VCAM-1 and the CS-1 peptide of fibronectin. *Exp. Cell Res.* 260, 73–84.
- Yu, Q., and Stamenkovic, I. (2000). Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF- $\beta$  and promotes tumor invasion and angiogenesis. *Genes Dev.* 14, 163–176.