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

Running title: Role of the MMP-9 target CD99 in CLL migration

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Abstract

We previously showed that MMP-9 contributes to CLL pathology by regulating cell survival and migration and that, when present at high levels, MMP-9 induces cell arrest. To further explore the latter function, we studied whether MMP-9 influences the gene expression profile in CLL. Microarray analyses rendered 131 differentially expressed genes in MEC-1 cells stably transfected with MMP-9 (MMP-9-cells) versus cells transfected with empty vector (Mock-cells). 10 out of 12 selected genes were also differentially expressed in MEC-1 cells expressing the catalytically inactive MMP-9MutE mutant (MMP-9MutE-cells). Incubation of primary CLL cells with MMP-9 or MMP-9MutE also regulated gene and protein expression, including CD99, CD226, CD52, and CD274. Because CD99 is involved in leukocyte transendothelial migration, we selected CD99 for functional and mechanistic studies. The link between MMP-9 and CD99 was reinforced with MMP-9 gene silencing studies, which resulted in CD99 upregulation. CD99 gene silencing significantly reduced CLL cell adhesion, chemotaxis and transendothelial migration, while CD99 overexpression increased cell migration. Mechanistic analyses indicated that MMP-9 downregulated CD99 via binding to $\alpha 4\beta 1$ integrin and subsequent inactivation of the Sp1 transcription factor. This MMP-9-induced mechanism is active in CLL lymphoid tissues, since CD99 expression and Sp1 phosphorylation was lower in bone marrow-derived CLL cells than in their peripheral blood counterparts. Our study establishes a new gene regulatory function for MMP-9 in CLL. It also identifies CD99 as an MMP-9 target and a novel contributor to CLL cell adhesion, migration and arrest. CD99 thus constitutes a new therapeutic target in CLL, complementary to MMP-9.

Keywords: Chronic lymphocytic leukemia; MMP-9; gene regulation; CD99; $\alpha4\beta1$ integrin; cell migration/arrest.

Introduction

Progression of chronic lymphocytic leukemia (CLL) is determined by infiltration of malignant cells into lymphoid organs.[1,2] Interaction with the microenvironment in these organs is beneficial for CLL cells, since they receive proliferative signals and acquire resistance to chemotherapy.[2] Several molecules regulate the migration and organ localization of these cells, including integrins, chemokines and matrix metalloproteinase-9 (MMP-9).[3]

CLL cells synthesize the proform and the activated form of MMP-9 (hereafter MMP-9 for either form).[4-6] Although mostly secreted into the medium, MMP-9 is also present at the CLL cell surface, where it binds to a $\alpha4\beta1$ integrin (CD49d/CD29)/$CD44_V$ complex.[4-7] We previously showed that MMP-9-$\alpha4\beta1$ integrin interaction contributes to CLL pathology, as it induces cell survival (by a non-catalytic mechanism) and regulates cell migration.[7-10] While constitutive MMP-9 is necessary for CLL cell migration, elevated levels of MMP-9 inhibit *in vitro* and *in vivo* migration, favoring cell arrest.[7,10] This was demonstrated with primary CLL cells incubated with MMP-9 and with MEC-1 cells stably transfected with empty vector (Mock-cells) or with MMP-9 (MMP-9-cells).[10] The mechanism accounting for the migration inhibitory effect of MMP-9 is not known, but it includes modulation of several molecules (RhoAGTPase, Akt, ERK, FAK, PTEN).[10] CLL cell-bound MMP-9 levels increase in lymphoid organs, likely because cells in the microenvironment

produce MMP-9 and several factors in these locations upregulate MMP-9 synthesis.[6,11,12]

We have also reported that incubation of CLL cells with a catalytically dead MMP-9 mutant (MMP-9MutE)[13] or transfection of MEC-1 cells with this mutant (MMP-9MutE-cells) partially affected signaling pathways and cell homing.[10,14] These previous results suggested that additional non-catalytic MMP-9 activities and local high MMP-9 expression contribute to CLL cell retention in lymphoid organs and disease progression. Non-proteolytic functions have also been reported for other MMPs, including MMP-1, MMP-2, MMP-3, MMP-12 and MMP-14.[15-18] These evidences prompted us to explore new functions of MMP-9 in CLL, particularly those that may contribute to malignant cell arrest in lymphoid niches.

In this report we performed gene expression analyses using MEC-1 cell transfectants and primary CLL cells. We show that MMP-9 regulates genes and proteins by means of catalytic and non-catalytic activities and we have focused on genes possibly involved in cell migration. We have identified CD99 as a new MMP-9 target and have determined the mechanism involved in its regulation by MMP-9. We further show that this mechanism is active in lymphoid tissues. Moreover, functional analyses revealed that CD99 is a novel molecule involved in CLL cell adhesion and migration, thus contributing to disease progression.

Results

MMP-9 regulates gene and protein expression in MEC-1 cells via catalytic and non-catalytic activities

To address the possibility that MMP-9 influences gene expression in CLL cells, we performed gene microarray analyses using total RNA from MEC-1 Mock-cells and MMP-9-cells. Initial analyses with normalized, unfiltered, values rendered 383 differentially expressed genes (172 up-regulated, 211 down-regulated) in MMP-9-cells, compared to Mock-cells (GSE78174). From these, we selected those genes whose expression change was ≥ 2-fold, resulting in 131 genes (35 upregulated and 96 downregulated) (Figure 1a and Supplementary Table S1). MMP-9 was the most upregulated gene (73-fold change), confirming the validity of the analysis. Functional allocation of these 131 genes was achieved using the DAVID database and the biological process (BP_FAT) category of Gene Ontology. Upon discarding non-significantly enriched processes, these analyses indicated that genes regulated by MMP-9 were mainly involved in intracellular signaling and immune response (16 genes each), cell adhesion (13 genes), cell motion (8 genes) and chemotaxis (5 genes), all potentially related to CLL pathology (Figure 1b). The specific genes allocated to each of these categories and their respective fold-change expression are listed in Supplementary Table S2.

To validate the microarray results, we randomly selected several genes among the most up-regulated or down-regulated (Supplementary Table S1) for quantitative PCR (qPCR) analyses. We also validated genes with known or potential functions in CLL, as well as genes involved in cell migration. These included: *CD226*, with a role in natural killer cell function in CLL;[19]

CD274, involved in CLL immune synapse;[20] *IL-10*, with immunoregulatory function and associated to CLL patient survival;[21] *CD52*, considered a CLL therapeutic target;[22] *CXCR3*, involved in CLL cell chemotaxis and with possible prognostic value;[23] and *CD99*, with a known role in leukocyte diapedesis but with no described function in CLL.[24-26] qPCR analyses confirmed the significant upregulation of *DDAH1, LPP* and *LRRC16A* and the significant downregulation of *CD99*, *CD226, STAP1, ADAM23, CD274*, *IL10, CD52, CCR8*, and *CXCR3* in MMP-9-cells, compared to Mock-cells (Figure 1c). To determine whether the MMP-9 enzymatic activity was required for the gene regulatory property we analyzed the expression of these genes in MMP-9MutE-cells, which carry the catalytically dead mutant MMP-9MutE.[13] Figure 1c shows that, with the exception of *CD52,* all other genes were significantly up or downregulated as in MMP-9-cells, albeit the extent of the effect was more limited in MMP-9MutE cells. Since MMP-9 and MMP-9MutE are expressed at similar levels in the transfectants,[14] the contribution of the catalytic domain may amplify the gene regulatory effect.

We then studied whether MMP-9 and MMP-9MutE also regulated protein expression. CD99, CD226, CD274, CD52 and CXCR3 were chosen for these studies, based on their mentioned possible relevance in CLL pathology. Because these proteins mainly function at the cell surface we first determined their expression by flow cytometry. Membrane expression of CD274 was very low in MEC-1 cells and no significant changes could be properly detected. Cell surface expression of CD99, CD226 and CXCR3 was significantly reduced in MMP-9-cells and, to a lesser extent, in MMP-9MutE-cells, compared to Mock-cells (Figure 1d). Surface expression of CD52 was

149 only significantly reduced in MMP-9-cells (Figure 1d), in agreement with the
150 qPCR results. Analysis of the total cellular levels of some of these proteins by
151 Western blotting showed the significant reduction of CD99 and CD274
152 expression in MMP-9-cells and MMP-9MutE-cells, compared to Mock-cells,
153 while CD52 was only reduced in MMP-9-cells (Figure 1e).
154
155 **MMP-9 and MMP-9MutE regulate gene and protein expression in primary**
156 **CLL cells**
157 We next studied whether MMP-9 and MMP-9MutE regulated gene expression
158 in primary CLL cells. CLL cells were incubated with these recombinant
159 proteins for 24 h and gene expression was analyzed by qPCR. As observed
160 for MEC-1 cells, MMP-9 upregulated *LPP* and *LRRC16A* and downregulated
161 *CD99, CD226, ADAM23, CD274* and *CD52*, while *DDHA1, STAP1, IL10,*
162 *CCR8 and CXCR3* were not differentially modulated (Figure 2a). MMP-9MutE
163 also significantly downregulated *CD99* and *CD274* but not the other genes*,*
164 compared to control cells (Figure 2a).
165 Analyses by flow cytometry indicated that incubation of CLL cells with
166 MMP-9 for 24 h or 48 h significantly reduced the cell surface expression of the
167 selected proteins CD99, CD226, CD274 and CD52, compared to control cells
168 (Figure 2b). Incubation with MMP-9MutE diminished the surface expression of
169 CD99, CD274 and CD52 (Figure 2b). Both MMP-9 and MMP-9MutE
170 significantly reduced the total cellular content of CD99, CD226 and CD274,
171 determined by Western blotting (Figure 2c). Collectively, these results
172 established that MMP-9 regulated gene and protein expression in MEC-1 cell

transfectants and primary CLL cells, by means of catalytic and/or non-catalytic activities.

Further analyses on the regulation of the MMP-9 target gene CD99

The GO analyses shown in Figure 1b indicated that many genes regulated by MMP-9 were related to cell adhesion and migration. Because we previously showed that elevated levels of MMP-9 impair CLL cell migration[7,10,14], we studied whether this impairment involved some of the newly identified MMP-9 target genes. Among these, CD99 is important for leukocyte extravasation[24-26] but its role in CLL is unknown. We thus selected CD99 for further functional and mechanistic studies.

To first confirm the interconnection between MMP-9 and CD99, we transfected MMP-9-cells with control or two MMP-9-specific siRNAs and measured the resulting levels of CD99. qPCR analyses confirmed that both siRNAs significantly reduced MMP-9 mRNA expression after 24 h, with values progressively recovering after 48 and 72 h (Figure 3a). Since MMP-9_1 siRNA was more efficient it was chosen for subsequent experiments. Gelatin zymography analyses indicated that MMP-9 levels were also reduced after 24 and 48 h (34% and 33%, respectively), compared to control cells (Figure 3b). In correlation with MMP-9 reduction, CD99 expression significantly increased after 48 h of *MMP9* silencing, both at the total cellular level (36%) (Figure 3b) and at the cell surface (18%) (Figure 3c). Importantly, transfection of primary CLL cells (3 patients) with MMP-9_1 siRNA significantly decreased (33%) MMP-9 mRNA after 24 h, with the concomitant increase (20%) of CD99 mRNA after 48 h (Figure 3d). At this time, the levels of MMP-9 protein were

198 also significantly reduced (65%) in the siRNA-transfected cells, with a parallel
199 increase on the expression of total (25%) (Figure 3e) and surface (14%)
200 (Figure 3f) CD99 protein.
201
202 **CD99-I is the major isoform expressed in MEC-1 cells and is involved in**
203 **transendothelial migration**
204 We next studied the possible involvement of CD99 in cell migration. Because
205 the reported CD99-I (32 kDa) and CD99-II (28 kDa) isoforms may play distinct
206 migratory roles in other cell systems,[26,27] we first examined the expression of
207 these isoforms in MEC-1 cells. qPCR analyses showed the expression of
208 *CD99-I* in Mock-cells and, at lower levels (0.2-fold less) in MMP-9-cells
209 (Figure 4a). In both cell types, *CD99-II* was hardly detected, while it was
210 present in monocytes, used as control for oligonucleotide validation (Figure
211 4a). In agreement with the results shown in Figures 1e and 3b with the DN16
212 antibody, additional analyses using the anti-CD99 antibody 12E7 confirmed
213 the presence of a single 32 kDa CD99 band in MEC-1-cells, while 12E7
214 recognized both CD99 isoforms in monocyte and Jurkat lysates (Figure 4b).
215 Therefore, subsequent studies were focused on CD99-I, hereafter called
216 CD99.
217 For functional studies, we transfected Mock-cells with two different
218 siRNAs for CD99. qPCR analyses indicated that both siRNAs reduced *CD99*
219 expression (87.4% and 57.4%, respectively) compared to control siRNA
220 values normalized to 1 (Figure 4c). CD99 protein was also reduced, both at
221 the total cellular level (Figure 4d) and at the cell surface (Figure 4e). CD99
222 downregulation diminished Mock-cell transendothelial migration in response

223 to CCL21 to 11.8% ($siRNA_1$) and 15.2% ($siRNA_2$), compared to the migration
224 of Mock-cells transfected with control siRNA (18.3%) (Figure 4f). To confirm
225 these results, we overexpressed CD99 in MMP-9-cells, since they have low
226 CD99 expression and impaired migration.[10,14] Transfection of MMP-9-cells
227 with a lentivirus containing CD99 cDNA increased CD99 surface expression
228 by 40%, compared to cells transfected with empty lentivirus (Figure 4g). CD99
229 overexpression significantly increased (36%) the transendothelial migration of
230 MMP-9-cells, compared to control cells (Figure 4h).
231
232 **Primary CLL cells predominantly express CD99-I and its downregulation**
233 **affects cell migration and adhesion**
234 Similar to MEC-1 cells, qPCR analyses of primary CLL cells demonstrated the
235 predominant expression of CD99-I and its downregulation by MMP-9 (Figure
236 5a), confirming the results shown in Figure 2c. CLL cells were transfected with
237 control siRNA or two CD99 siRNAs and analyzed by Western blotting and
238 flow cytometry. These analyses confirmed the significant reduction of CD99 at
239 the total cellular level (33.5% and 24.9%, respectively, for $CD99_1$ and $CD99_2$)
240 (Figure 5b) and at the cell surface (35.6% and 21%, respectively) (Figure 5c).
241 Both CD99 siRNAs reduced CLL transendothelial migration, compared to
242 cells transfected with control siRNA (35% and 27.4% reduction for $CD99_1$ and
243 $CD99_2$, respectively) (Figure 5d). The role of CD99 in CLL cell migration was
244 further confirmed by performing chemotaxis assays, which showed that both
245 CD99 siRNAs diminished (52% and 42%, respectively) CLL cell chemotaxis in
246 response to CCL21 (Figure 5e).

In other cell systems, CD99 was shown to modulate integrin-mediated cell adhesion.[27,28] To determine if CD99 performed this function in CLL, we first analyzed the adhesion of Mock-cells and MMP-9-cells to VCAM-1 and FN-89, two $\alpha4\beta1$ integrin ligands[6]. Figure 5f shows that MMP-9-cells (low CD99 expression) displayed significantly lower adhesion to these substrates than Mock-cells. In agreement with this, silencing *CD99* in primary CLL cells significantly reduced cell adhesion to VCAM-1 and FN-H89, compared to cells transfected with control siRNA (Figure 5g). Collectively, the gene silencing and overexpression experiments clearly demonstrated the novel role of CD99 in CLL cell migration and adhesion.

CD99 downregulation requires binding of MMP-9 to $\alpha4\beta1$ integrin

We next studied the mechanism involved in the regulation of CD99 by MMP-9. Because binding of MMP-9 (or MMP-9MutE) to $\alpha4\beta1$ integrin induces survival signalling in CLL cells[8], we determined whether regulation of gene expression by MMP-9 also involved $\alpha4\beta1$. CLL cells (6 patients, >30% $\alpha4$ integrin expression) were treated or not with MMP-9 and CD99 expression (mRNA and protein) upon incubation with MMP-9 was normalized to 1. Blocking the binding of MMP-9 to $\alpha4\beta1$ integrin with the HP2/1 mAb prevented the downregulation of CD99 mRNA (Figure 6a) and CD99 surface expression (Figure 6b), observed in the absence of HP2/1. In both cases, the Ig isotype control had no effect.

To confirm these results, we transfected CLL cells with two *$\alpha4$* integrin-specific siRNAs. Both siRNAs significantly reduced *$\alpha4$* expression (average 37% and 34% reduction, respectively), measured by qPCR 48 h after

transfection, compared to cells transfected with control siRNA (Figure 6c). Surface expression of α4 integrin at this time was also significantly decreased by both siRNAs (Figure 6d). The expression of cell-bound MMP-9 on cells transfected with α4 or control siRNAs was also analyzed without (endogenous MMP-9) or with incubation with MMP-9, and values (% positive cells) for control siRNA-transfected cells in each case were normalized to 1. *α4* silencing significantly decreased the constitutive levels of membrane-bound MMP-9 to 0.43 ($\alpha4_1$) and 0.33 ($\alpha4_2$), compared to their corresponding control (Figure 6e). Upon incubation with MMP-9, these levels were also significantly lower (0.76 and 0.64, respectively, for $\alpha4_1$ and $\alpha4_2$ siRNAs) than in control cells (Figure 6e).

We next determined whether *α4* silencing and reduced membrane-bound MMP-9 affected CD99 expression. qPCR analyses demonstrated that CD99 mRNA was higher on *α4* silenced-cells, both without (1.35-fold and 1.55-fold, respectively, for $\alpha4_1$ and $\alpha4_2$) or with incubation with MMP-9 (1.42-fold for $\alpha4_1$ and 1.43-fold for $\alpha4_2$), compared to their corresponding control (Figure 6f). Cell surface expression of CD99 was also significantly higher in *α4*-silenced-cells, in the absence (1.37-fold and 1.46-fold for $\alpha4_1$ and $\alpha4_2$, respectively) or presence (1.35-fold and 1.20-fold, respectively) of exogenous MMP-9 (Figure 6g). These results indicated that MMP-9 regulated CD99 expression via binding to $\alpha4\beta1$ integrin at the CLL cell membrane.

MMP-9 downregulates CD99 via Sp1 inactivation

The preceding results indicated that MMP-9 regulated CD99 at the transcriptional level. Because in previous reports it was shown that Sp1 is the

major inducer of CD99 expression in cancer cell lines,[29,30] we studied whether Sp1 regulated CD99 in CLL cells. In initial experiments, we incubated primary CLL cells with the Sp1 inhibitor mithramycin and measured CD99 expression after 24 h. An NF-κB inhibitor was also included as control in these experiments. Mithramycin significantly and nearly completely reduced CD99 mRNA expression in a concentration-dependent manner (Figure 7a). Reduction at the protein level was moderate but also significant, perhaps reflecting a higher stability of CD99 at the cell surface (Figure 7b). The NF-κB inhibitor did not affect CD99 expression (Figure 7a, b).

We next determined whether MMP-9 regulated CD99 via Sp1. Incubation of primary CLL cells with MMP-9 significantly diminished Sp1 phosphorylation, measured after 2 and 24 h, but had no effect on phospho-p65 levels (Figure 7c). To complement these results, we transfected CLL cells with MMP-9_1 siRNA or a control siRNA and measured the levels of phopho-Sp1 and phospho-p65 after 24 h of transfection. Gene silencing MMP-9 significantly increased phospho-Sp1, without affecting phospho-p65 (Figure 7d). Gene silencing α4 integrin also significantly increased phospho-Sp1, measured 48 h after transfection with the specific siRNA, while the levels of phospho-p65 did not change (Figure 7e). Altogether, these results demonstrated that binding of MMP-9 to α4β1 integrin induced Sp1 dephosphorylation, leading to CD99 downregulation.

CLL cells present in bone marrow have lower CD99 surface expression and Sp1 phosphorylation than their peripheral blood counterparts

Subsequently, we studied whether downregulation of CD99 occurred in the pathophysiological context of CLL. Initial examination of the constitutive expression of surface MMP-9 and CD99 revealed no direct correlation between both proteins (Supplementary Table S3). Because CLL cells present in bone marrow (BM) express more cell-bound MMP-9 than their peripheral blood (PB) counterparts,[8] we reasoned that CD99 expression would be reduced in BM-derived cells. To address this, CLL cells from BM and PB of the same individuals were analyzed by flow cytometry. We first confirmed in the six patients studied that BM cells displayed higher expression of surface MMP-9 than their PB counterparts (Figure 8a). In correlation with this, CD99 expression was significantly lower (average 24.5%) in BM cells than in PB cells (Figure 8b). In accordance with the reduced CD99 expression, BM cells had significantly lower levels of phospho-Sp1 than PB cells, while the levels of phospho-p65 were similar in both cell types (Figure 8c). These results clearly established that the outline mechanism of CD99 downregulation by elevated levels of MMP-9 is active in CLL.

Discussion

To better understand the contribution of MMP-9 to CLL progression, particularly its role in cell migration/arrest, we have studied whether MMP-9 modulated gene expression. Our major findings are: 1) MMP-9 affects the transcriptional profile of CLL cells; 2) CD99 is an MMP-9 target and a novel contributor to CLL cell migration and adhesion; 3) CD99 downregulation requires MMP-9 binding to $\alpha 4\beta 1$ integrin and Sp1 inactivation; 4) This regulatory mechanism is active in the CLL BM.

346 Gene expression analyses indicated that MMP-9 affects the expression
347 of many genes in CLL cells. These genes were mainly allocated to immune
348 response, intracellular signaling, and cell adhesion/migration functions. This is
349 in agreement with the known role of MMP-9 in CLL cell migration,[5-7,10,12] and
350 with its strong association with immune functions.[31,32] The gene regulatory
351 effect of MMP-9 was partly observed in MMP-9Mut-cells and in primary CLL
352 cells incubated with MMP-9MutE, indicating that this function involved only in
353 part the MMP-9 catalytic activity. Accordingly, CD99, CD226, CD274, CXCR3,
354 or CD52 were not identified as direct MMP-9 substrates in a degradome
355 analysis by quantitative proteomics.[33] In general, however, MMP-9 was more
356 efficient than MMP-9MutE, both at the gene and protein level regulation. This
357 is consistent with the reported partial effect of MMP-9MutE in the *in vitro* and
358 *in vivo* migration of CLL cells and MMP-9MutE-cells,[10,14] and suggests the
359 contribution of several MMP-9 regions, one of them being the catalytic
360 domain, to fully achieve these functions.
361 A crucial finding in our study is that the results obtained in the model
362 system of MEC-1 cell transfectants were reproduced by incubating primary
363 CLL cells with MMP-9 and, for some genes/proteins, with MMP-9MutE. The
364 MMP-9 gene regulatory effect was clearly significant in CLL cells but more
365 limited than in MEC-1 cells. These quantitative differences might be explained
366 in the context of the two cell systems used. Stable transfection of MMP-9 in
367 MEC-1 cells may provide a sustained stimulus and in a homogenous cell
368 population, while in primary CLL cells the response to MMP-9 is likely
369 heterogeneous and more moderate. The selected genes/proteins regulated in
370 primary CLL cells were CD226, CD274 CD52, and CD99, the first three with

371 known functions in CLL.[19,20,34] To date, no role has been attributed to CD99 in
372 CLL and we have chosen this molecule for further studies concerning its
373 regulation by MMP-9.

374 CD99 is a transmembrane protein with two isoforms, I and II, which
375 arise by alternative splicing of the CD99 gene.[26,27] The expression of these
376 isoforms is cell-type specific and CD99-I is the predominant form in
377 hematopoietic cells.[26,35,36] In agreement with this, CLL cells mainly expressed
378 CD99-I, while CD99-II was barely detected. In some cell systems, CD99
379 isoforms have opposite functions in cell migration and adhesion, two closely
380 related pocesses.[26] For example, CD99-I and CD99-II inhibited or induced,
381 respectively, osteosarcoma cell migration by inversely modulating c-Src
382 activity[37] and inhibiting ROCK2.[38] However, CD99-I also supports cell
383 migration, having a well-characterized role in leukocyte transendothelial
384 migration, where it functions sequentially after PECAM1 and forms a signaling
385 complex with soluble adenyl cyclase, PKA and ezrin.[24-26] Indeed, CD99-I
386 induced migration in monocytes,[39] CD34+ cells,[40] and malignant glioma
387 cells,[41] indicating a dual role for CD99-I, likely depending on the cell context.
388 Our present results are the first to demonstrate a supportive role for CD99-I in
389 CLL cell migration.

390 CD99 was also shown to modulate $\alpha 4\beta 1$ integrin binding to VCAM-1
391 and increased T cell adhesion to endothelium.[28] Likewise, CD99 upregulated
392 the LFA-1/ICAM-1 interaction, inducing B and T cell homotypic adhesion[27,36]
393 or neutrophil arrest in venules.[42] The adhesion of glioma cells to laminin was
394 also regulated by CD99.[41] We now show that gene silencing CD99 diminished
395 CLL cell adhesion to the $\alpha 4\beta 1$ integrin ligands VCAM-1 and FN-H89,

396 establishing a novel role for CD99 in the regulation of integrin function in CLL.
397 Other reported properties for CD99 include its role CD99 in apoptosis and
398 lymphocyte development.[26,35] CD99 was proposed to be a marker for minimal
399 residual disease in acute lymphoblastic leukemia.[43] In solid tumors, CD99
400 may be expressed at high (Ewing sarcoma, B-cell lymphoma) or low levels
401 (Hodgkin´s lymphoma, gastric carcinoma), the latter suggesting an
402 oncosuppressor function.[35] Modulation of CD99 expression, as we observe in
403 our study, may therefore have important consequences for malignant cells.
404 Future studies should determine whether CD99 is involved in other
405 pathological processes in CLL

406 We have addressed the mechanism by which MMP-9 regulates CD99.
407 Our results show that this regulation required MMP-9 binding to $\alpha4\beta1$ integrin
408 at the cell surface. We previously reported that the MMP-9-$\alpha4\beta1$ integrin
409 interaction induces survival signaling[8] and impairs *in vitro* and *in vivo* CLL cell
410 migration, by affecting migration regulatory pathways.[7,8] Both of these
411 functions involved MMP-9 catalytic and non-catalytic activities. We now
412 expand these studies and report a novel signaling MMP-9 function, also
413 elicited upon binding to CLL cells via $\alpha4\beta1$ integrin, consisting in the
414 regulation of CD99 expression. CD99 is therefore part of the mechanism by
415 which MMP-9 impairs CLL cell migration.

416 Our results further demonstrate that downregulation of CD99 involves
417 inactivation of the Sp1 transcription factor. Sp1 was shown to positively
418 regulate CD99 in lymphoma, embryonic kidney cells, and gastric
419 carcinoma.[29,30] The transcriptional activity of Sp1 is tightly regulated, being
420 influenced by its phosphorylation state, other post-translational modifications,

421 and/or interaction with other nuclear factors, which may induce or repress
422 Sp1-mediated transcription.[44,45] In our study, the following evidences support
423 the involvement of Sp1 in CD99 downregulation by MMP-9: 1) The Sp1
424 inhibitor mithramycin reduced CD99 expression in a dose-dependent manner;
425 2) Sp1 phosphorylation was constitutively lower in MEC-1 MMP-9-cells than in
426 Mock-cells and was significantly decreased in primary CLL cells upon
427 incubation with MMP-9; 3) Gene silencing α4 integrin or MMP-9 significantly
428 increased Sp1 phosphorylation and CD99 cell surface expression. Sp1
429 inactivation is therefore a novel consequence of MMP-9 binding to $\alpha4\beta1$
430 integrin in CLL cells. The fact that Sp1 also regulates genes involved in
431 proliferation/survival, angiogenesis and stress response[45] highlights the
432 relevance of the MMP-9-$\alpha4\beta1$ integrin interaction in CLL cells.

433 It is now demonstrated that elevated α4 integrin expression (>30%)
434 constitutes an unfavorable prognostic marker in CLL.[46] $\alpha4\beta1$ integrin induces
435 cell survival, drug resistance and is required for CLL cell homing to BM, all
436 contributing to disease progression.[3,6,8,47] Our present results demonstrate
437 that the MMP-9-$\alpha4\beta1$-induced CD99 regulation is active in the
438 pathophysiological context of CLL. This was not inferred from examination of
439 MMP-9 and CD99 expression in PB CLL cells, since no correlation was
440 observed in all cases. Therefore, the constitutive MMP-9 synthesized or
441 bound by circulating CLL cells may not be sufficient to impact on gene/protein
442 expression. We previously showed[8] and confirmed here that CLL cells
443 isolated from lymphoid organs have higher membrane-bound MMP-9 than
444 their PB counterparts, reflecting the higher MMP-9 levels present in these
445 niches. Consistent with the increased MMP-9 expression, BM-derived CLL

446 cells expressed lower CD99 and phospho-Sp1 levels than PB-derived cells
447 from the same individual. These findings unequivocally demonstrate that
448 downregulation of CD99 by elevated levels of MMP-9 is actively induced in
449 BM as a CLL niche.

450 Our results are in accordance with previous gene expression analyses
451 (dataset GSE21029) showing lower *CD99* expression in BM-derived CLL cells
452 than in PB-CLL cells.[48] This was not the case for *CD99* expression in lymph
453 node-derived CLL cells, indicating mechanistic differences likely due to the
454 distinct molecular signatures observed among CLL tissues.[49] MMP-9
455 regulation of CD99 may thus be an important molecular process for CLL cells
456 in the BM. Because we previously showed that high MMP-9 expression
457 impairs CLL cell migration,[7,10] downregulation of CD99 may represent a
458 critical mechanism controlling CLL cell traffic and arrest. CLL cells entering
459 BM would be exposed to high concentrations of MMP-9 which, upon binding
460 to $\alpha 4\beta 1$ integrin, would downregulate CD99 and favor retention in this organ.
461 Because cells in the BM receive survival and proliferative signals, regulation
462 of CD99 by MMP-9 may directly impact CLL progression. This cell arrest
463 effect is in sharp contrast with an old dogma stating that MMPs are purely
464 stimulators of cancer cell invasion and metastasis.

465 In summary, our study is the first to demonstrate that MMP-9 regulates
466 gene and protein expression in CLL. It also identifies CD99 as an MMP-9
467 target and a novel contributor to CLL cell migration and retention in the BM.
468 MMP-9 and CD99 may therefore represent therapeutic targets in CLL.
469
470

Materials and Methods

Patients, cells and cell cultures

Approval was obtained from the CSIC Bioethics Review Board for these studies. Peripheral blood (PB) samples from the 39 untreated CLL patients listed in Supplementary Table S3 were obtained after informed consent. B-lymphocytes were purified by Ficoll-Paque™ Plus (GE Healthcare Europe GmbH, Barcelona, Spain) centrifugation and the resulting cell population was mostly >90% $CD19^+$, determined on a Coulter Epics XL flow cytometer (Beckman Coulter, Fullerton, CA). Some paired CLL samples from PB and BM were obtained from Dr. Dolors Colomer (Hospital Clinic, Barcelona, Spain). MEC-1 cells were purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and authenticated by DNA profiling. MEC-1 cells stably transfected with empty vector (Mock-cells), MMP-9 (MMP-9-cells) or catalytically inactive MMP-9MutE (MMP-9MutE-cells) were generated as described[10,14] and maintained in IMDM medium (Lonza, Basel, Switzerland), 10% FBS. Human umbilical vein endothelial cells (HUVEC) were purchased from Lonza and cultured in EGM™ Endothelial Cell Growth Medium BulletKit™ (Lonza).

Cell adhesion assays

These assays were performed on 96-well plates coated with 0.5% BSA or 2.5 µg/ml VCAM-1 or FN-H89[6]. 10^5 MEC-1 or primary CLL cells were incubated with 1.4 ng/ml 2,7-bis(carboxyethyl)-5(6)-carboxyfluoresceinacetoxymethyl ester (BCECF-AM, Molecular Probes, Eugene, OR) for 30 min, suspended in RPMI 1640, 0.5% BSA, and added to the coated wells. After 45 min at 37 °C,

attached cells were lysed with PBS, 0.1% SDS and quantified using a fluorescence analyzer (BMG Labtech, Offenburg, Germany).

Cell migration assays

For chemotaxis assays, $3x10^5$ cells in medium were added to the upper chamber of Transwell filters (Costar, New York, NY) and allowed to migrate towards medium containing 200 ng/ml CCL21 in the lower chamber. After 24 h at 37°C, migrated cells were counted by flow cytometry. For transendothelial migration, $7.5x10^4$ HUVEC were plated on fibronectin-coated (10 μg/ml) Transwell filters and confluent monolayers were stimulated with 15 ng/ml TNF-α for 16 h before the assay. CLL cells ($3x10^5$) were added to the HUVEC monolayer and transmigration towards medium containing CCL21 was monitored after 24 h as above. Cells that migrated in both types of assays were expressed as percentage of the total number of cells added, also counted by flow cytometry.

Conflict of interest

The authors declare no conflict of interest

Acknowledgements

The authors thank Dr. Dolors Colomer for some of the paired CLL samples; Dr. Miguel A. Vega for expert help and advice with the microarray data analyses; Dr. Pedro Lastres for help with the flow cytometry analyses; and Guillermo Padilla for expert help with bioinformatics analyses.

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Author contributions

NAM and EB performed most of the research, designed experiments and analyzed data; RUC performed research and analyzed data; AS, AGG and CPS performed and analyzed some experiments; EUB designed and prepared cell transfectants and analyzed data; GO and PEVdS prepared and characterized the recombinant MMP-9 variants and critically reviewed the manuscript; JAGM contributed patient samples, with clinical, biological and cytogenetic data; AGP designed and supervised research, had full access to the data and wrote the paper. All authors reviewed and approved the final version of the manuscript.

Supplementary information is available at the Oncogene´s website (http://www.nature.com/onc).

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710

711 **Figure legends**

712 **Figure 1.** Regulation of gene and protein expression in MEC-1 cells
713 transfected with MMP-9 or MMP-9MutE. (**a**) Heat map representing color-
714 coded expression levels of 131 differently expressed genes in MMP-9-cells
715 and Mock-cells. Analyses were performed in four (Mock-cells) or five (MMP-9-
716 cells) different samples. Details of regulated genes are provided in
717 Supplementary Table S1. (**b**) Functional annotation of the 131 genes shown

718 in (**a**) using the BP_FAT category of GO and the DAVID database. (**c**) qPCR
719 validation of 12 selected genes in MMP-9-cells and MMP-9MutE-cells, after
720 normalizing the value of each gene in Mock-cells to 1. TATA-binding protein
721 (TBP) expression was used as an internal control and average values are
722 shown. Ctrol, control. (**d**) Flow cytometry determination of the cell surface
723 expression of the indicated proteins in Mock-, MMP-9-, and MMP-9MutE-cells.
724 (**e**) The total cellular expression of the indicated proteins was analyzed by
725 Western blotting, after loading 25 μg protein and using vinculin as internal
726 control. Values compared for statistical significance in panels c-e are those of
727 MMP-9- or MMP-9MutE-cells *versus* the values of Mock-cells for each
728 individual gene. Ctrol, control; MFI, mean fluorescence intensity; *P < 0.05;
729 **P < 0.01; ***P < 0.001.
730
731 **Figure 2.** MMP-9 and MMP-9MutE regulate gene and protein expression in
732 primary CLL cells. (**a**) qPCR analyses of the indicated selected genes in
733 primary CLL cells after incubation without (control, Ctrol) or with 110 nM
734 MMP-9 or MMP-9MutE for 24 h. The values of control cells for each gene
735 were normalized to 1. Average values from three or four different patients are
736 shown. (**b**) Flow cytometry determination of the cell surface expression of the
737 indicated proteins in CLL cells treated as in (**a**). (**c**) Cells treated as above
738 were lysed and 25 μg protein/condition were loaded and analyzed by Western
739 blotting, using vinculin as internal control. Values compared for statistical
740 significance in all panels are those of CLL cells incubated with MMP-9 or
741 MMP-9MutE *versus* the values of CLL cells incubated in medium alone

742 (control, Ctrol). MFI, mean fluorescence intensity; *P < 0.05; **P < 0.01; ***P
743 < 0.001.
744
745 **Figure 3.** MMP-9 gene silencing upregulates CD99 expression in MEC-1 (**a-**
746 **c**) and primary (**d-f**) CLL cells. (**a**) $15x10^6$ MMP-9-cells were transfected with
747 control or two different MMP-9 siRNAs and MMP-9 mRNA expression was
748 analyzed by qPCR. The average values of 3 different experiments are shown.
749 (**b**) The MMP-9_1 siRNA-transfected cells shown in (**a**) were analyzed by
750 gelatin zymography (MMP-9) and Western blotting (CD99). The results from a
751 representative experiment and the average quantitation of the 3 experiments
752 performed are shown. (**c**) Surface expression of CD99 on the same MMP-9-
753 silenced cells shown in (**a**-**b**), measured by flow cytometry. (**d**) qPCR
754 analyses showing the expression of MMP-9 and CD99 upon transfection of
755 $15x10^6$ CLL cells (3 patients) with control or MMP-9_1 siRNA. (**e**) Gelatin
756 zymography of conditioned medium (MMP-9) and Western blotting analyses
757 of lysates (CD99) of CLL cells after 48 h of transfection with MMP-9_1 siRNA.
758 The results from a representative patient and the average quantitation of the 3
759 patients analyzed are shown. (**f**) CD99 surface expression on the same cells
760 shown in (**e**) analyzed by flow cytometry. 25 μg protein/condition were loaded
761 for the Western blotting analyses. Values compared for statistical significance
762 are those of MMP-9-cells (panels a-c) or of primary CLL cells (d-f) *versus* their
763 respective control. FC, fold change; Ctrol, control; MFI, mean fluorescence
764 intensity; Ctrol, control; *P < 0.05; **P < 0.01; ***P < 0.001.
765

766 **Figure 4.** CD99 is involved in MEC-1 cell transendothelial migration. (**a**)
767 Constitutive expression of *CD99-I* and *CD99-II* isoforms in Mock and MMP-9-
768 expressing MEC-1 cell transfectants measured by qPCR. Monocytes
769 (Monoc.) were used as positive control for *CD99-II* expression. (**b**) Western
770 blotting analyses of the constitutive expression of CD99 isoforms in Mock-
771 cells and MMP-9-cells, using the 12E7 anti-CD99 antibody. Lysates of
772 monocytes and Jurkat cells were used as positive controls for CD99-II (28
773 kDa). Protein load: 25 µg. CD99-I values in Mock-cells were normalized to 1.
774 (**c-e**) $15x10^6$ Mock-cells were transfected with the indicated CD99 siRNAs and
775 the efficiency of the transfection monitored after 48 h by qPCR (**c**), Western
776 blotting (**d**) and flow cytometry (**e**). (**f**) Mock-cells, transfected with the
777 indicated siRNAs for 48 h were added to the upper chamber of Transwell
778 filters coated with HUVEC and allowed to migrate in response to CCL21 (200
779 ng/ml) for 20 h. Numbers represent the percentage of migrated cells,
780 determined by flow cytometry. (**g**) $2x10^6$ MMP-9-cells were infected with
781 lentiviral particles (LV) alone (Ctrol) or containing CD99-I/CD99-II cDNA, and
782 CD99 expression analyzed after 72 h by flow cytometry. (**h**) The
783 transendothelial migration of LV-infected MMP-9-cells, in response to CCL21,
784 was determined by flow cytometry. Ctrol, control; MFI, mean fluorescence
785 intensity. *P < 0.05; **P < 0.01; ***P < 0.001.
786
787 **Figure 5.** CD99 is involved in primary CLL cell migration and adhesion. (**a**)
788 qPCR analysis of the expression of *CD99* isoforms in primary CLL cells (four
789 patients), incubated without (control, Ctrol) or with MMP-9 for 24 h. (**b-c**) 15-
790 $30x10^6$ primary CLL cells (seven patients) were transfected with the indicated

siRNAs and, after 48 h, CD99 expression was analyzed by Western blotting (**b**) and flow cytometry (**c**). Values were obtained after normalizing control values to 1. (**d-e**) The transendothelial migration (**d**) and chemotaxis (**e**), in response to CCL21, of CLL cells transfected with the indicated siRNAs was measured by flow cytometry. Average values obtained with CLL cells of seven (**d**) or four (**e**) different patients are shown. (**f)** 10^5 Mock-cells and MMP-9 cells were labeled with BCECF-AM and added to 96-well plates coated with 2.5 μg/ml VCAM-1 or FN-H89. After 60 min, cell adhesion was measured using a fluorescence analyzer. (**g**) The adhesion of 10^5 primary CLL cells (four patients), transfected with the indicated siRNAs, to FN-H89 or VCAM-1 was analyzed as explained in (**f**). Protein load for Western blotting: 25 μg; Ctrol, control; MFI, mean fluorescence intensity. *P < 0.05; **P < 0.01; ***P < 0.001.

Figure 6. Downregulation of CD99 by MMP-9 requires binding to α4β1 integrin. (**a**-**b**) CLL cells (6 patients) were treated or not with the HP2/1 anti-α4 mAb or an isotype control Ig. After 24 h, CD99 mRNA (**a**) and cell surface expression (**b**) was determined by qPCR and flow cytometry, respectively. (**c**-**d**) CLL cells (3 patients) were transfected with the indicated siRNAs and the efficiency of the transfection was determined after 48 h by qPCR (**c**) and flow cytometry (**d**). (**e**) MMP-9 surface expression on CLL cells transfected with the indicated siRNAs, with or without incubation with MMP-9 for the last 24 h. (**f**-**g**) CD99 mRNA (**f**) and cell surface expression (**g**) was determined on the same cells shown in (**e**) by qPCR and flow cytometry, respectively. Values compared for statistical significance in panels c-g are those of CLL cells transfected with the indicated siRNAs versus cells transfected with control

siRNA. FC, fold change; MFI, mean fluorescence intensity; Ctrol, control; *P < 0.05; **P < 0.01; ***P < 0.001.

Figure 7. MMP-9 downregulates CD99 via Sp1 inactivation. $5x10^6$ CLL cells were treated with the Sp1 inhibitor mithramycin (Mit) or an NF-κB inhibitor at the indicated doses. After 24 h CD99 mRNA (**a**) and cell surface expression (**b**) was determined. (**c**) Primary CLL cells (four patients) were incubated or not with MMP-9 for the indicated times and the phosphorylation of Sp1 (at T453) and p65 (at S536) was measured by Western blotting. (**d-e**) CLL cells were transfected with MMP-9_1 (**d**) or $\alpha4_1$ (**e**) siRNAs and the phosphorylation of Sp1 and p65 was measured by Western blotting. 25 μg protein/condition were loaded for Western blotting analyses. Values compared for statistical significance are those of CLL cells under the various conditions versus their respective control. FC, fold change; MFI, mean fluorescence intensity; Ctrol, control; *P < 0.05; **P < 0.01; ***P < 0.001.

Figure 8. CD99 downregulation by MMP-9 is active in the CLL bone marrow. (**a**) Surface-bound MMP-9 on CLL cells isolated from peripheral blood (PB) and bone marrow (BM) from six different patients, after normalizing PB values to 1. (**b**) Flow cytometry images and average quantitation of CD99 expression from PB (grey lines) and BM (red lines). Numbers indicate MFI values. (**c**) Western blotting analyses (30 μg protein/sample) of Sp1 and p65 phosphorylation in CLL cells from PB and BM. Average BM values after normalizing the respective PB values to 1 are shown. MFI, mean fluorescence intensity; *P < 0.05; **P < 0.01.

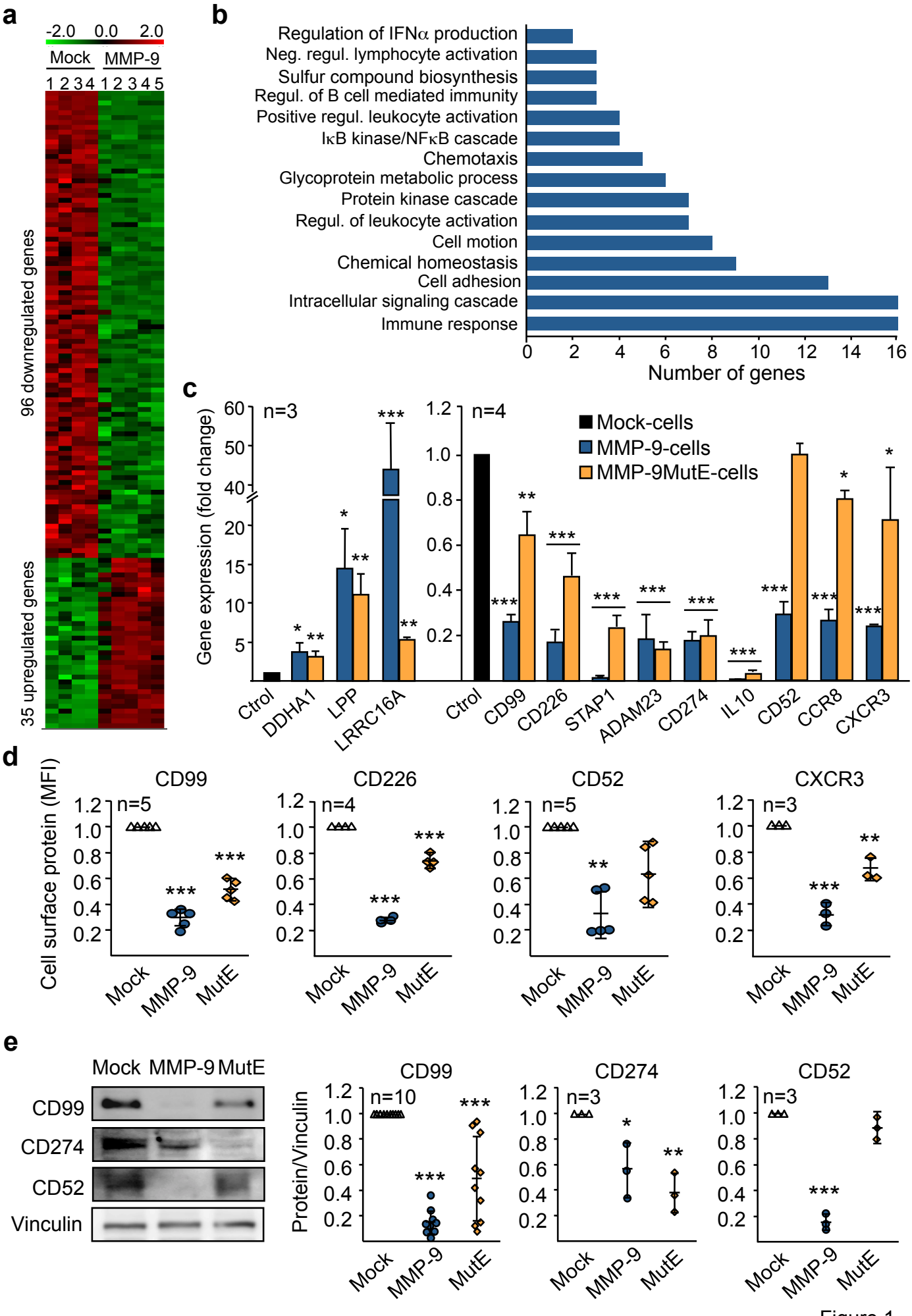
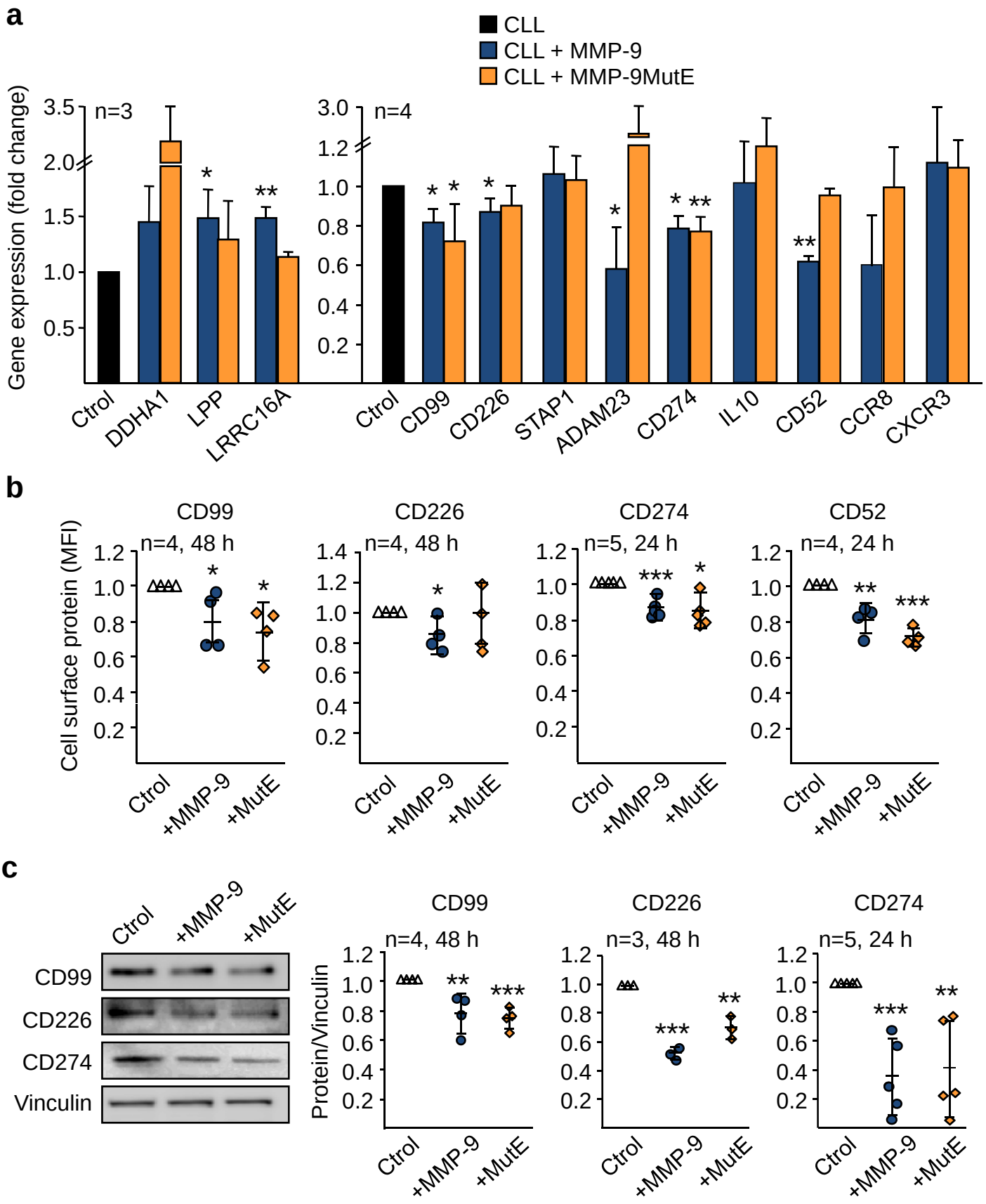

a
-2.0 0.0 2.0
Mock MMP-9
1 2 3 4 1 2 3 4 5
96 downregulated genes
35 upregulated genes
b
Regulation of IFNα production
Neg. regul. lymphocyte activation
Sulfur compound biosynthesis
Regul. of B cell mediated immunity
Positive regul. leukocyte activation
IκB kinase/NFκB cascade
Chemotaxis
Glycoprotein metabolic process
Protein kinase cascade
Regul. of leukocyte activation
Cell motion
Chemical homeostasis
Cell adhesion
Intracellular signaling cascade
Immune response
0 2 4 6 8 10 12 14 16
Number of genes
c
Gene expression (fold change)
n=3
n=4
Mock-cells
MMP-9-cells
MMP-9MutE-cells
Ctrol DDHA1 LPP LRRC16A
Ctrol CD99 CD226 STAP1 ADAM23 CD274 IL10 CD52 CCR8 CXCR3
d
Cell surface protein (MFI)
CD99
CD226
CD52
CXCR3
n=5
n=4
n=5
n=3
Mock MMP-9 MutE
e
Mock MMP-9 MutE
CD99
CD274
CD52
Vinculin
Protein/Vinculin
CD99
CD274
CD52
n=10
n=3
n=3
Mock MMP-9 MutE

Figure 1


a
CLL
CLL + MMP-9
CLL + MMP-9MutE
Gene expression (fold change)
n=3
n=4
Ctrol
DDHA1
LPP
LRRC16A
CD99
CD226
STAP1
ADAM23
CD274
IL10
CD52
CCR8
CXCR3
b
Cell surface protein (MFI)
CD99
n=4, 48 h
CD226
n=4, 48 h
CD274
n=5, 24 h
CD52
n=4, 24 h
Ctrol
+MMP-9
+MutE
c
Ctrol
+MMP-9
+MutE
CD99
CD226
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CD226
n=3, 48 h
CD274
n=5, 24 h

Figure 2

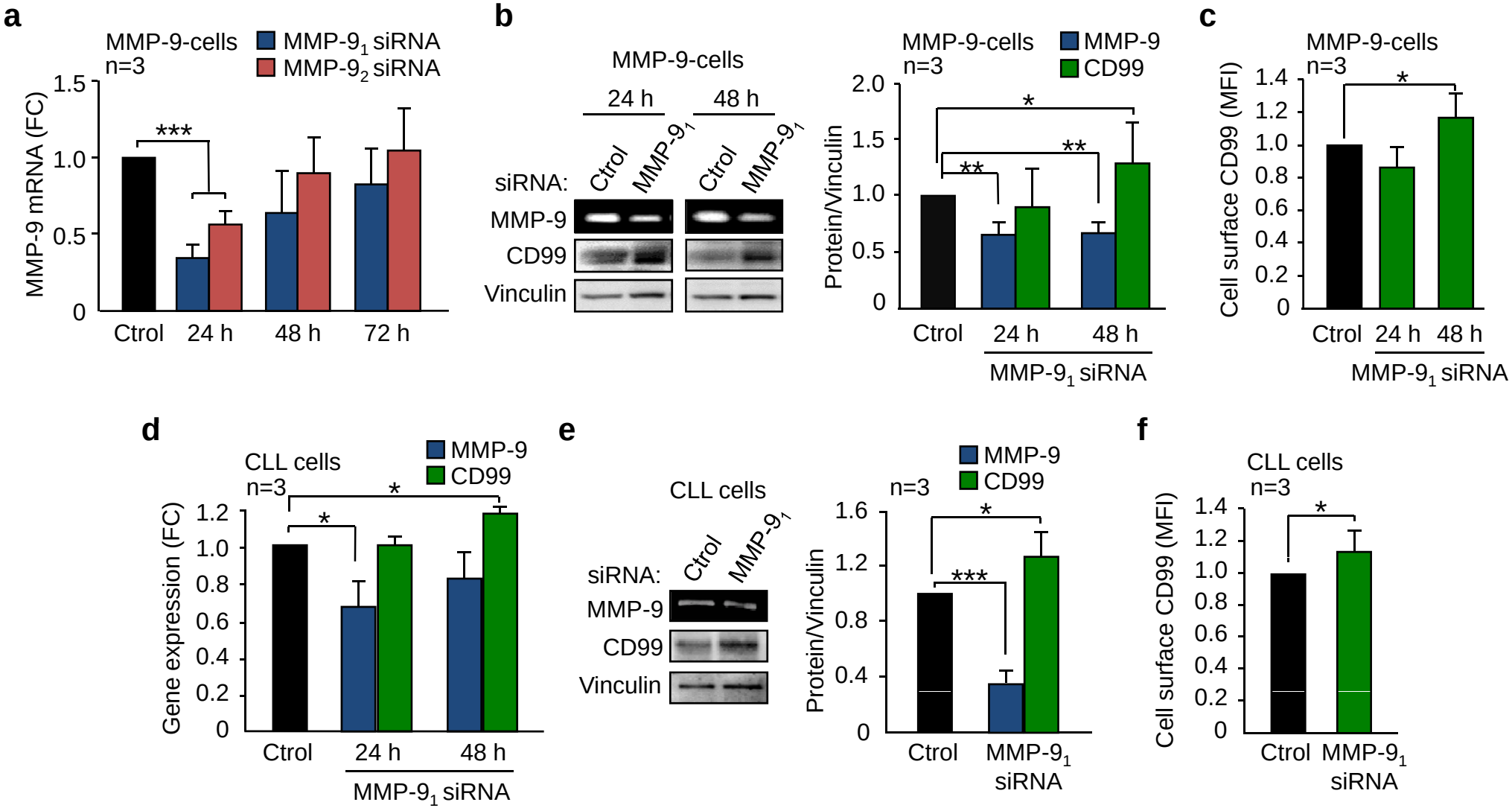
a
MMP-9-cells
n=3
MMP-9$_1$ siRNA
MMP-9$_2$ siRNA
MMP-9 mRNA (FC)
1.5
1.0
0.5
0
***
Ctrol
24 h
48 h
72 h
b
MMP-9-cells
24 h
48 h
siRNA:
Ctrol
MMP-9$_1$
Ctrol
MMP-9$_1$
MMP-9
CD99
Vinculin
MMP-9-cells
n=3
MMP-9
CD99
Protein/Vinculin
2.0
1.5
1.0
0.5
0
*
**
**
Ctrol
24 h
48 h
MMP-9$_1$ siRNA
c
MMP-9-cells
n=3
Cell surface CD99 (MFI)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
*
Ctrol
24 h
48 h
MMP-9$_1$ siRNA
d
CLL cells
n=3
MMP-9
CD99
Gene expression (FC)
1.2
1.0
0.8
0.6
0.4
0.2
0
*
*
Ctrol
24 h
48 h
MMP-9$_1$ siRNA
e
CLL cells
siRNA:
Ctrol
MMP-9$_1$
MMP-9
CD99
Vinculin
n=3
MMP-9
CD99
Protein/Vinculin
1.6
1.2
0.8
0.4
0
*
***
Ctrol
MMP-9$_1$
siRNA
f
CLL cells
n=3
Cell surface CD99 (MFI)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
*
Ctrol
MMP-9$_1$
siRNA

Figure 3

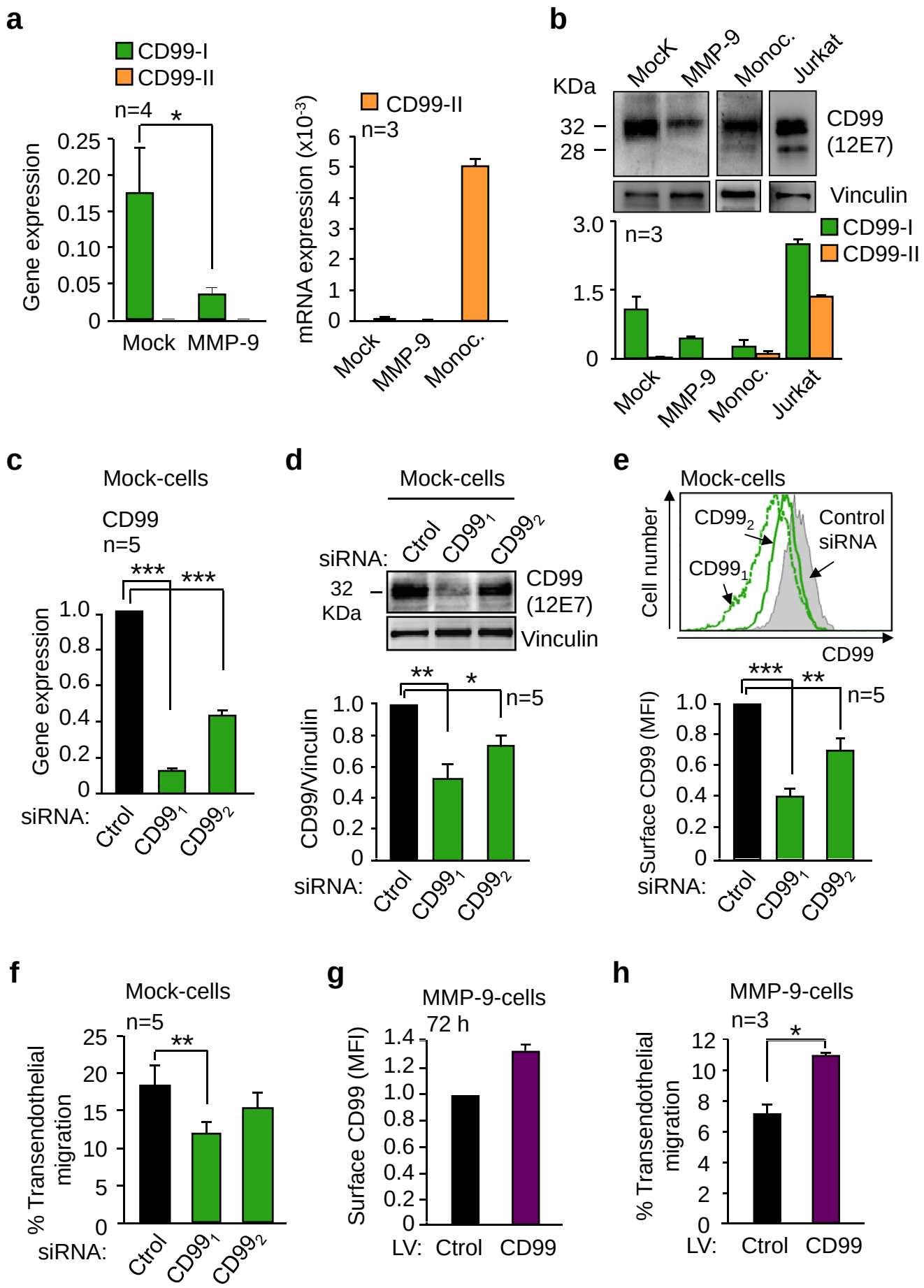
a
CD99-I
CD99-II
n=4
*
Gene expression
0.25
0.20
0.15
0.10
0.05
0
Mock
MMP-9
CD99-II
n=3
mRNA expression (x10-3)
6
5
4
3
2
1
0
Mock
MMP-9
Monoc.
b
Mock
MMP-9
Monoc.
Jurkat
KDa
32
28
CD99
(12E7)
Vinculin
n=3
CD99-I
CD99-II
3.0
1.5
0
Mock
MMP-9
Monoc.
Jurkat
c
Mock-cells
CD99
n=5
***
***
Gene expression
1.0
0.8
0.6
0.4
0.2
0
siRNA:
Ctrol
CD991
CD992
d
Mock-cells
siRNA:
Ctrol
CD991
CD992
32
KDa
CD99
(12E7)
Vinculin
**
*
n=5
CD99/Vinculin
1.0
0.8
0.6
0.4
0.2
0
siRNA:
Ctrol
CD991
CD992
e
Mock-cells
Cell number
CD992
Control
siRNA
CD991
CD99
***
**
n=5
Surface CD99 (MFI)
1.0
0.8
0.6
0.4
0.2
0
siRNA:
Ctrol
CD991
CD992
f
Mock-cells
n=5
**
% Transendothelial
migration
25
20
15
10
5
0
siRNA:
Ctrol
CD991
CD992
g
MMP-9-cells
72 h
Surface CD99 (MFI)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
LV:
Ctrol
CD99
h
MMP-9-cells
n=3
*
% Transendothelial
migration
12
10
8
6
4
2
0
LV:
Ctrol
CD99

Figure 4

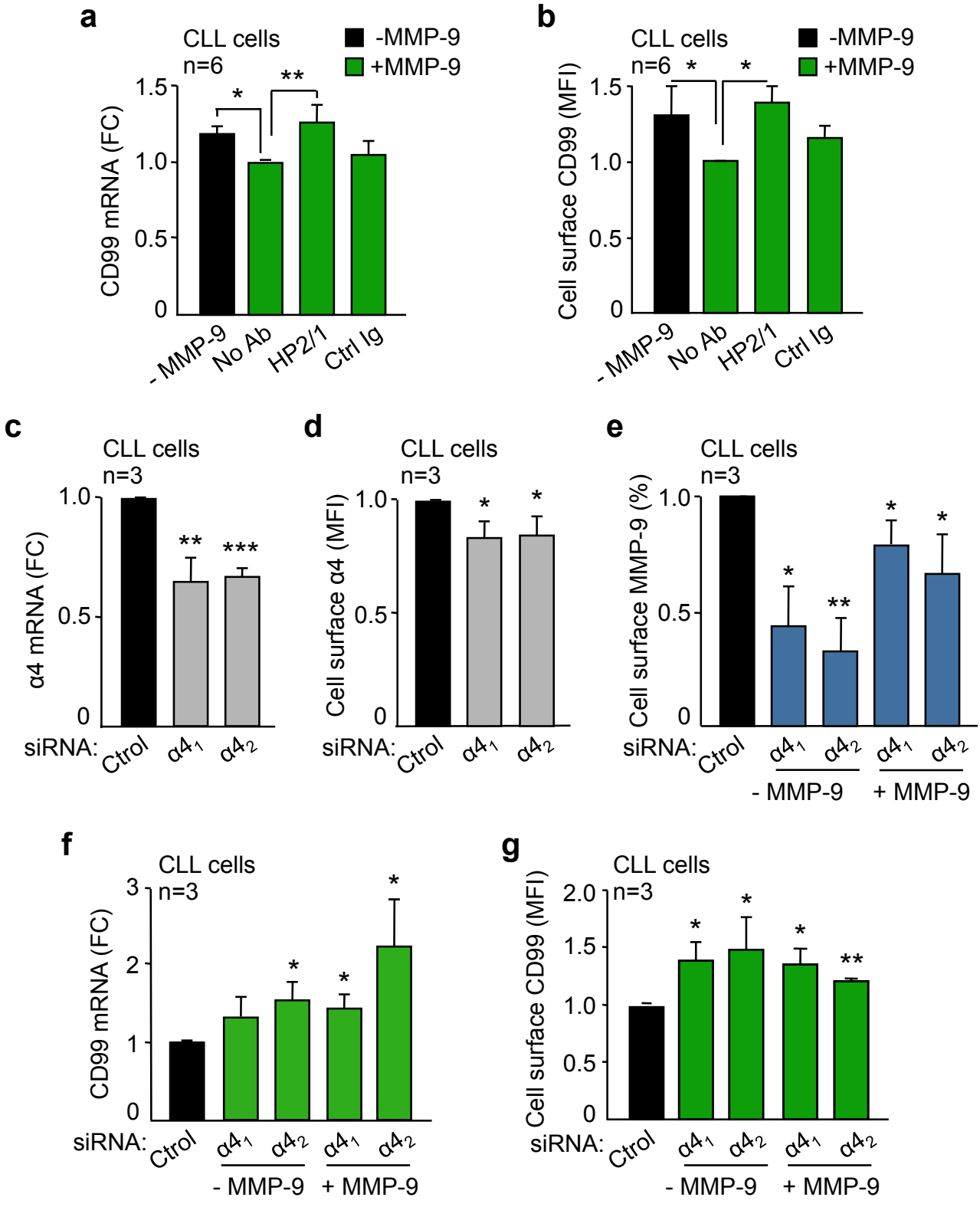
a
CLL cells
n=6
-MMP-9
+MMP-9
CD99 mRNA (FC)
1.5
1.0
0.5
0
*
**
- MMP-9
No Ab
HP2/1
Ctrl Ig
b
CLL cells
n=6
-MMP-9
+MMP-9
Cell surface CD99 (MFI)
c
CLL cells
n=3
α4 mRNA (FC)
**
***
siRNA:
Ctrol
α4₁
α4₂
d
CLL cells
n=3
Cell surface α4 (MFI)
e
CLL cells
n=3
Cell surface MMP-9 (%)
- MMP-9
+ MMP-9
f
CLL cells
n=3
CD99 mRNA (FC)
3
2
1
g
CLL cells
n=3
Cell surface CD99 (MFI)
2.0

Figure 5

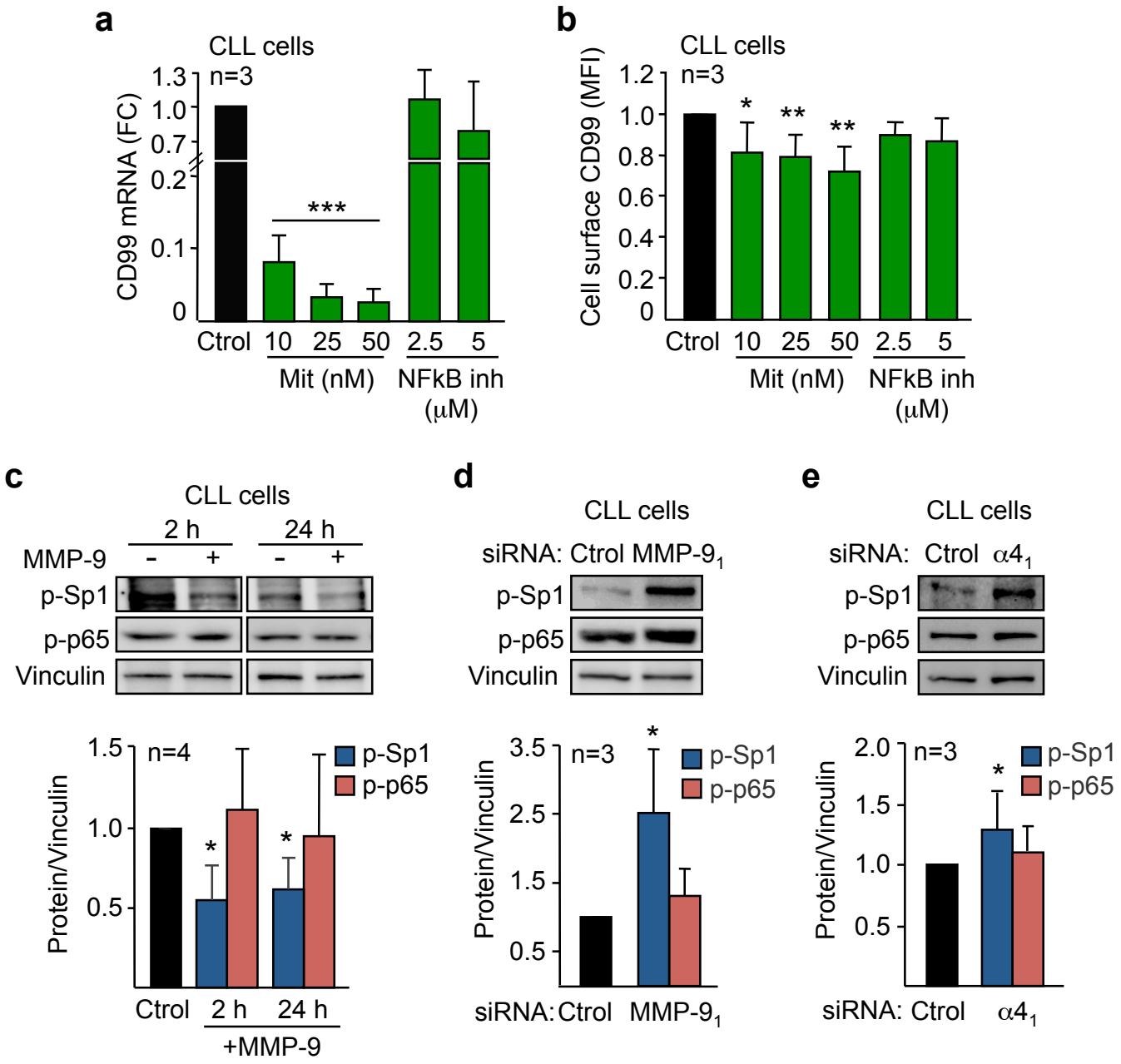
a
CLL cells
n=3
CD99 mRNA (FC)
1.3
1.0
0.7
0.2
0.1
0
***
Ctrol
10
25
50
2.5
5
Mit (nM)
NFkB inh (μM)
b
CLL cells
n=3
Cell surface CD99 (MFI)
1.2
1.0
0.8
0.6
0.4
0.2
0
*
**
**
Ctrol
10
25
50
2.5
5
Mit (nM)
NFkB inh (μM)
c
CLL cells
2 h
24 h
MMP-9
-
+
-
+
p-Sp1
p-p65
Vinculin
n=4
p-Sp1
p-p65
Protein/Vinculin
1.5
1.0
0.5
*
*
Ctrol
2 h
24 h
+MMP-9
d
CLL cells
siRNA: Ctrol MMP-9$_1$
p-Sp1
p-p65
Vinculin
n=3
p-Sp1
p-p65
Protein/Vinculin
3.5
2.5
1.5
0.5
*
siRNA: Ctrol
MMP-9$_1$
e
CLL cells
siRNA: Ctrol α4$_1$
p-Sp1
p-p65
Vinculin
n=3
p-Sp1
p-p65
Protein/Vinculin
2.0
1.5
1.0
0.5
*
siRNA: Ctrol
α4$_1$

Figure 6

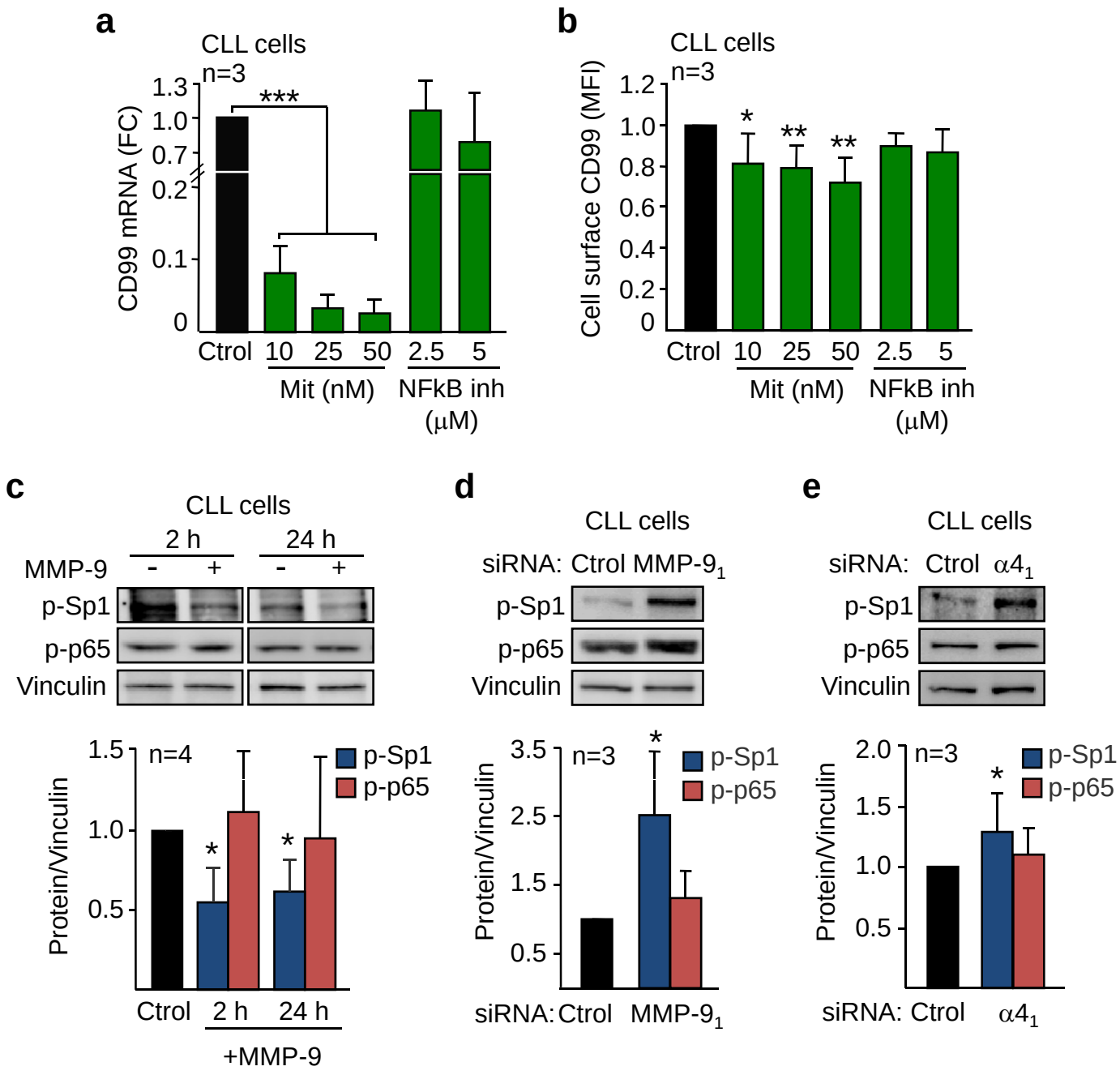
a
CLL cells
n=3
CD99 mRNA (FC)
1.3
1.0
0.7
0.2
0.1
0
***
Ctrol
10
25
50
2.5
5
Mit (nM)
NFkB inh (μM)
b
CLL cells
n=3
Cell surface CD99 (MFI)
1.2
1.0
0.8
0.6
0.4
0.2
0
*
**
**
Ctrol
10
25
50
2.5
5
Mit (nM)
NFkB inh (μM)
c
CLL cells
2 h
24 h
MMP-9
-
+
-
+
p-Sp1
p-p65
Vinculin
n=4
p-Sp1
p-p65
Protein/Vinculin
1.5
1.0
0.5
*
*
Ctrol
2 h
24 h
+MMP-9
d
CLL cells
siRNA: Ctrol MMP-9$_1$
p-Sp1
p-p65
Vinculin
n=3
p-Sp1
p-p65
Protein/Vinculin
3.5
2.5
1.5
0.5
*
siRNA: Ctrol
MMP-9$_1$
e
CLL cells
siRNA: Ctrol α4$_1$
p-Sp1
p-p65
Vinculin
n=3
p-Sp1
p-p65
Protein/Vinculin
2.0
1.5
1.0
0.5
*
siRNA: Ctrol
α4$_1$

Figure 7

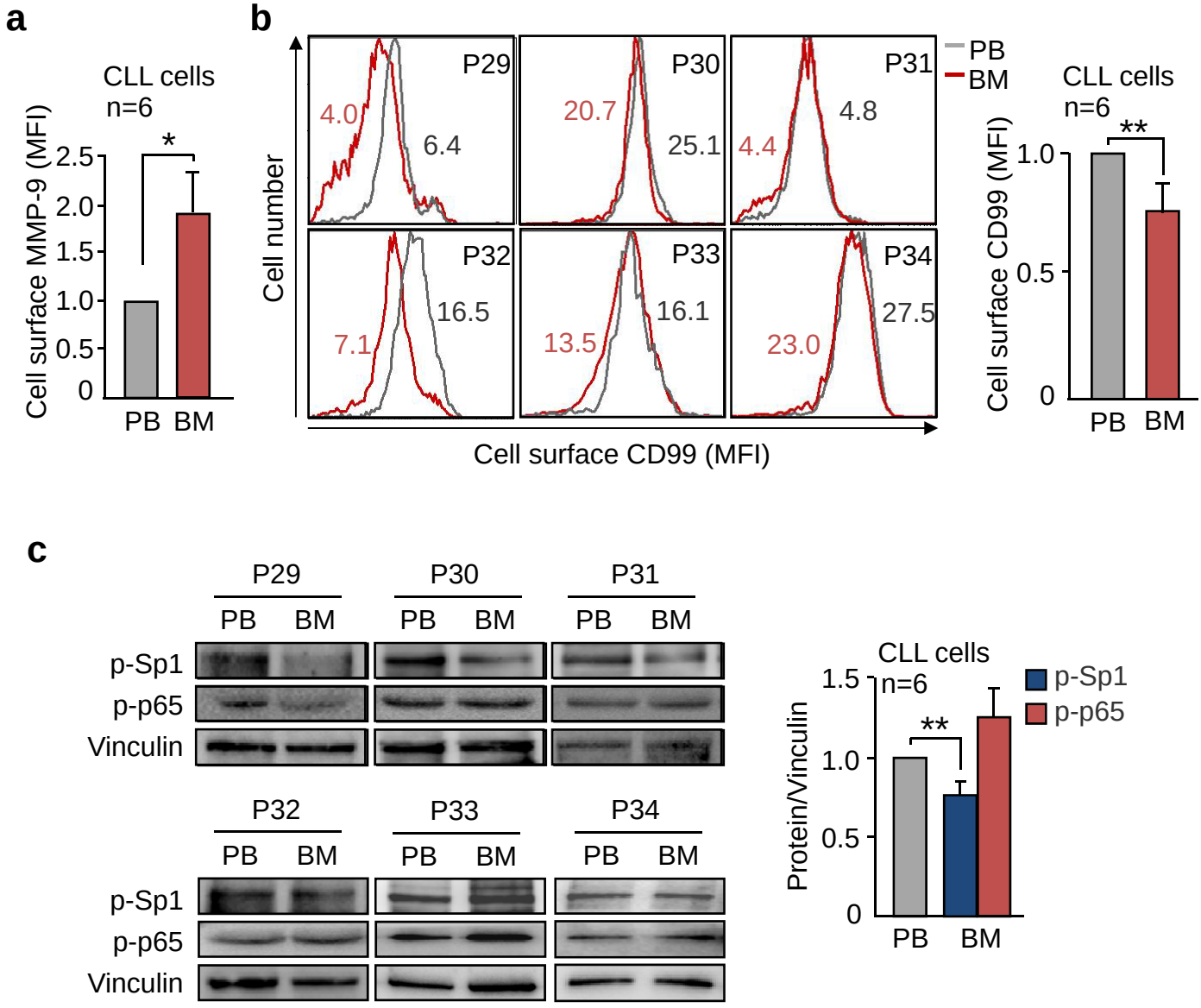
a
CLL cells
n=6
Cell surface MMP-9 (MFI)
2.5
2.0
1.5
1.0
0.5
0
*
PB
BM
b
Cell number
P29
4.0
6.4
P30
20.7
25.1
P31
4.4
4.8
P32
7.1
16.5
P33
13.5
16.1
P34
23.0
27.5
PB
BM
Cell surface CD99 (MFI)
CLL cells
n=6
Cell surface CD99 (MFI)
1.0
0.5
0
**
PB
BM
c
P29
P30
P31
PB
BM
p-Sp1
p-p65
Vinculin
P32
P33
P34
CLL cells
n=6
Protein/Vinculin
1.5
1.0
0.5
0
**
p-Sp1
p-p65
PB
BM

Figure 8

Aguilera-Montilla et al – Supplementary Information

Supplementary Table S1. Significantly modulated genes (96 downregulated, 35 upregulated) in MMP-9-cells corresponding to the heat map shown in Figure 1a.

Gene name	Functional description	R-fold
EIF1AY	Eukaryotic translation initiation factor 1A, Y-linked	0.028
RPS4Y1	Ribosomal protein S4, Y-linked 1	0.053
CD226	CD226 molecule	0.055
DDX3Y	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked	0.062
MAP7D3	MAP7 domain containing 3	0.066
STAP1	Signal transducing adaptor family member 1	0.089
UTY	Ubiquitously transcribed tetratricopeptide repeat gene, Y-linked	0.095
ADAM23	ADAM metallopeptidase domain 23	0.106
CYorf15A	Chromosome Y open reading frame 15A	0.116
CCR8	Chemokine (C-C motif) receptor 8	0.122
ZNF286	Zinc finger protein 286A	0.128
GNG11	Guanine nucleotide binding protein (G protein), gamma 11	0.151
KIR3DX1	Killer cell immunoglobulin-like receptor, three domains, X1	0.158
GNA15	Guanine nucleotide binding protein (G protein), alpha 15 (Gq class)	0.166
CLEC17A	C-type lectin domain family 17, member A	0.169
ZFY	Zinc finger protein, Y-linked	0.174
CCND2	Cyclin D2	0.179
TFPI2	Tissue factor pathway inhibitor 2	0.180
NRG4	Neuregulin 4	0.186
TLR7	Toll-like receptor 7	0.189
TM4SF19	Transmembrane 4 L six family member 19	0.195
FGFR1	Fibroblast growth factor receptor 1	0.203
COTL1	Coactosin-like 1	0.207
NLGN4Y	Neuroligin 4, Y-linked	0.218
CCL1	Chemokine (C-C motif) ligand 1	0.219
PARP15	Poly (ADP-ribose) polymerase family, member 15	0.219
SERPINB9	Serpin peptidase inhibitor, clade B (ovalbumin), member 9	0.227
PRF1	Perforin 1 (pore forming protein)	0.237
GPR141	G protein-coupled receptor 141	0.240
CYorf15B	Chromosome Y open reading frame 15B	0.243
CXCR3	Chemokine (C-X-C motif) receptor 3	0.245
CD99	CD99 molecule	0.245
CYSLTR1	Cysteinyl leukotriene receptor 1	0.247
SLC37A2	Solute carrier family 37 (glycerol-3-phosphate transporter) member 2	0.247
HS3ST3B1	Heparan sulfate (glucosamine) 3-O-sulfotransferase 3B1	0.249
SPIC	Spi-C transcription factor (Spi-1/PU.1 related)	0.249
KDM5D	Lysine (K)-specific demethylase 5D	0.249
ACY3	Aspartoacylase (aminocyclase) 3	0.252
ZNF462	Zinc finger protein 462	0.259
PAG1	Phosphoprotein associated with glycosphingolipid microdomains 1	0.262
EPAS1	Endothelial PAS domain protein 1	0.266
CXorf57	Chromosome X open reading frame 57	0.270
CD274	CD274 molecule	0.277
RAB11FIP1	RAB11 family interacting protein 1 (class I)	0.282
USP9Y	Ubiquitin specific peptidase 9, Y-linked	0.283
KLHL13	Kelch-like 13 (Drosophila)	0.287

NID1	Nidogen 1	0.289
SPIB	Spi-B transcription factor (Spi-1/PU.1 related)	0.293
NRCAM	Neuronal cell adhesion molecule	0.294
ZNF532	Zinc finger protein 532	0.299
VCAM1	Vascular cell adhesion molecule 1	0.312
IL10	Interleukin 10	0.312
FAM174B	Family with sequence similarity 174, member B	0.316
C11orf63	Uncharacterized protein C11orf63	0.316
FEZ1	Fasciculation and elongation protein zeta 1 (zygin I)	0.332
SORBS2	Sorbin and SH3 domain containing 2	0.335
FCGR2B	Fc fragment of IgG, low affinity IIb, receptor (CD32)	0.337
IGF1	Insulin-like growth factor 1 (somatomedin C)	0.346
TLE1	Transducin-like enhancer of split 1 (E(sp1) homolog, Drosophila)	0.354
TMEM2	Transmembrane protein 2	0.379
ABCG1	ATP-binding cassette, sub-family G (WHITE), member 1	0.387
RASA3	RAS p21 protein activator 3	0.387
CLNK	Cytokine-dependent hematopoietic cell linker	0.396
GPR174	G protein-coupled receptor 174	0.398
GPM6A	Glycoprotein M6A	0.398
CD52	CD52 molecule	0.401
KHDRBS3	KH domain containing, RNA binding, signal transduction associated 3	0.405
FLT1	Fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor)	0.411
EEPD1	Endonuclease/exonuclease/phosphatase family domain containing 1	0.411
CASK	Calcium/calmodulin-dependent serine protein kinase (MAGUK family)	0.413
OAS3	2'-5'-oligoadenylate synthetase 3, 100kDa	0.416
UGCG	UDP-glucose ceramide glucosyltransferase	0.423
POU2F2	POU class 2 homeobox 2	0.424
ANO5	Anoctamin 5	0.425
GAS7	Growth arrest-specific 7	0.426
RBPMS2	RNA binding protein with multiple splicing 2	0.427
NCF4	Neutrophil cytosolic factor 4, 40kDa	0.427
BCL2A1	BCL2-related protein A1	0.434
TYROBP	TYRO protein tyrosine kinase binding protein	0.436
ARHGAP20	Rho GTPase activating protein 20	0.437
NAPSB	Napsin B aspartic peptidase pseudogene	0.440
CSF2RB	Colony stimulating factor 2 receptor, beta, low-affinity (granulocyte-macrophage)	0.445
SLC16A7	Solute carrier family 16, member 7 (monocarboxylic acid transporter 2)	0.454
ARAP2	ArfGAP with RhoGAP domain, ankyrin repeat and PH domain 2	0.455
TBX21	T-box 21	0.456
CALHM2	Calcium homeostasis modulator 2	0.463
TNFSF14	Tumor necrosis factor (ligand) superfamily, member 14	0.469
MAN1A1	Mannosidase, alpha, class 1A, member 1	0.470
MGAT4A	Mannosyl (alpha-1,3-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase, isozyme A	0.472
FAM134B	Family with sequence similarity 134, member B	0.479
RNF125	Ring finger protein 125	0.481
TSPAN7	Tetraspanin 7	0.497
WDR17	WD repeat domain 17	0.498
TSPAN5	Tetraspanin 5	0.501
C6orf174	KIAA0408	0.507
TSPAN6	Tetraspanin 6	0.508
ZNF610	Zinc finger protein 610	2.059
ZMAT1	Zinc finger, matrin type 1	2.097

LY96	Lymphocyte antigen 96	2.116
TMEM145	Transmembrane protein 145	2.129
PDE4D	Phosphodiesterase 4D, cAMP-specific (phosphodiesterase E3 dunce homolog, Drosophila)	2.150
TMEM173	Transmembrane protein 173	2.333
HBE1	Hemoglobin, epsilon 1	2.373
PITPNC1	Phosphatidylinositol transfer protein, cytoplasmic 1	2.440
DNAJC12	DnaJ (Hsp40) homolog, subfamily C, member 12	2.443
RBM9	RNA binding motif protein 9	2.446
MAPK10	Mitogen-activated protein kinase 10	2.460
ZNF682	Zinc finger protein 682	2.464
ZNF544	Zinc finger protein 544	2.466
FHIT	Fragile histidine triad gene	2.500
GIMAP6	GTPase, IMAP family member 6	2.503
VWDE	Von Willebrand factor D and EGF domains	2.573
PIK3R5	Phosphoinositide-3-kinase, regulatory subunit 5	2.590
ACSM3	Acyl-CoA synthetase medium-chain family member 3	2.707
NRXN3	Neurexin 3	2.746
IGHG1	Immunoglobulin heavy constant gamma 1	3.153
TP53INP1	Tumor protein p53 inducible nuclear protein 1	3.357
AC008620.3	Olfactory receptor 2V1	3.505
SMAD9	SMAD family member 9	4.296
LPP	LIM domain containing preferred translocation partner in lipoma	4.414
MT1F	Metallothionein 1F	4.428
PFN2	Profilin 2	4.482
CBS	Cystathionine-beta-synthase	4.718
SERINC2	Serine incorporator 2	4.874
DDAH1	Dimethylarginine dimethylaminohydrolase 1	5.093
TRPS1	Trichorhinophalangeal syndrome I	5.326
RAVER2	Ribonucleoprotein, PTB-binding 2	5.398
ST6GALNAC2	ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 2	6.406
LRRC16A	Leucine rich repeat containing 16A	6.499
ZNF717	Zinc finger protein 717	8.080
MMP9	Matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	73.094

Supplementary Table S2. Biological process allocation of significantly regulated genes in MMP-9-cells compared to Mock-cells. See also Figure 1b.

Function	Symbol	Description	P value	Fold change
Immune response GO: 0006955			0.0001	
	IGHG1	Immunoglobulin heavy constant gamma 1		3.152
	TMEM173	Transmembrane protein 173		2.332
	LY96	Lymphocyte antigen 96		2.116
	RNF125	Ring finger protein 125		0.481
	TNFSF14	Tumor necrosis factor (ligand) superfamily, member 14		0.469
	NCF4	Neutrophil cytosolic factor 4, 40kDa		0.427
	POU2F2	POU class 2 homeobox 2		0.423
	OAS3	2'-5'-oligoadenylate synthetase 3, 100kDa		0.415
	CLNK	Cytokine-dependent hematopoietic cell linker		0.396
	FCGR2B	Fc fragment of IgG, low affinity IIb, receptor (CD32)		0.336
	IL10	Interleukin 10		0.312
	CD274	CD274 molecule		0.276
	PAG1	Phosphoprotein associated with glycosphingolipid microdomains 1		0.262
	CCL1	Chemokine (C-C motif) ligand 1		0.218
	TLR7	Toll-like receptor 7		0.189
	CCR8	Chemokine (C-C motif) receptor 8		0.122
Intracellular signaling cascade GO: 0007242			0.033	
	DDAH1	Dimethylarginine dimethylaminohydrolase 1		5.092
	MAPK10	Mitogen-activated protein kinase 10		2.460
	RBM9	RNA binding motif protein 9		2.445
	LY96	Lymphocyte antigen 96		2.116
	TNFSF14	Tumor necrosis factor (ligand) superfamily, member 14		0.469
	TYROBP	TYRO protein tyrosine kinase binding protein		0.435
	FLT1	Fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor)		0.410
	CLNK	Cytokine-dependent hematopoietic cell linker		0.396
	RASA3	RAS p21 protein activator 3		0.387
	IGF1	Insulin-like growth factor 1 (somatomedin C)		0.346
	IL10	Interleukin 10		0.312
	PAG1	Phosphoprotein associated with glycosphingolipid microdomains 1		0.262
	FGFR1	Fibroblast growth factor receptor 1		0.202
	TLR7	Toll-like receptor 7		0.189
	GNA15	Guanine nucleotide binding protein (G protein), alpha 15 (Gq class)		0.165
	GNG11	Guanine nucleotide binding protein (G protein), gamma 11		0.151

Cell adhesion GO:0007155			0.0027	
	LPP	LIM domain containing preferred translocation partner in lipoma		4.413
	NRXN3	Neurexin 3		2.745
	CASK	Calcium/calmodulin-dependent serine protein kinase (MAGUK family)		0.413
	FEZ1	Fasciculation and elongation protein zeta 1		0.332
	VCAM1	Vascular cell adhesion molecule 1		0.312
	NRCAM	Neuronal cell adhesion molecule		0.294
	NID1	Nidogen 1		0.289
	CD99	CD99 molecule		0.245
	CXCR3	Chemokine (C-X-C motif) receptor 3		0.245
	NLGN4Y	Neuroligin 4, Y-linked		0.218
	CCR8	Chemokine (C-C motif) receptor 8		0.122
	ADAM23	ADAM metallopeptidase domain 23		0.106
	CD226	CD226 molecule		0.054
Cell motion GO: 0006928			0.0542	
	NRXN3	Neurexin 3		2.745
	FLT1	Fms-related tyrosine kinase 1 (VEGF receptor)		0.410
	IGF1	Insulin-like growth factor 1 (somatomedin C)		0.346
	FEZ1	Fasciculation and elongation protein zeta 1		0.332
	IL10	Interleukin 10		0.312
	VCAM1	Vascular cell adhesion molecule 1		0.312
	NRCAM	Neuronal cell adhesion molecule		0.294
	CXCR3	Chemokine (C-X-C motif) receptor 3		0.245
Chemotaxis GO: 0006935			0.0279	
	IL10	Interleukin 10		0.312
	CYSLTR1	Cysteinyl leukotriene receptor 1		0.247
	CXCR3	Chemokine (C-X-C motif) receptor 3		0.245
	CCL1	Chemokine (C-C motif) ligand 1		0.218
	CCR8	Chemokine (C-C motif) receptor 8		0.122

Supplementary Table S3. Clinical characteristics of CLL patients

Patient	Sex/ Age	Stage[a]	Ig Status[b]	CD38/ ZAP70	α4[c]	MMP-9[c]	CD99[c]
1	M/73		M	+/+	88.5 (2.5)	ND	99.2 (49.5)
2	M/79	B/II	U	+/-	47.3 (8.0)	6.6 (0.8)	ND
3	M/86	A/I	ND	-/ND	39.8 (1.6)	4.4 (2.1)	ND
4	F/88	B/II	M	-/+	93.4 (4.1)	ND	99.8 (19.5)
5	M/48	B/I	U	+/+	48.9 (2.1)	5.0 (0.6)	ND
6	F/70	C/IV	ND	ND	80.7 (2.2)	ND	ND
7	F/55	B/II	M	+/+	98.7 (7.9)	7.7 (0.5)	ND
8	M/80	C/IV	M	-/-	94.1 (2.4)	ND	99.4 (8.7)
9	M/75		M	+/-	45.6 (0.9)	15.6 (0.5)	99.0 (15.5)
10	F/46	B/II	U	+/+	54.3 (1.0)	10.7 (0.4)	99.6 (7.9)
11	M/47	B/II	M	+/+	78.4 (1.9)	4.5 (0.4)	ND
12	F/59	B/II	U	-/+	95.7 (2.8)	16.2 (0.5)	99.3 (10.4)
13	M/47	B/II	M	+/+	36.3 (0.4)	ND	99.7 (17.1)
14	M/70	B/II	U	-/+	45.3 (0.6)	ND	99.8 (7.5)
15	F/88	B/II	U	+/-	61.1 (1.2)	2.6 (0.6)	99.2 (32.4)
16	F/86	C/III	U	+/ND	62.1 (0.5)	5.4 (0.4)	98.0 (12.8)
17	F/54	B/II	M	-/ND	56.9 (1.1)	12.4 (0.5)	98.5 (6.5)
18	M/75	B/II	U	+/ND	75.7 (2.3)	12.6 (0.4)	99.4 (9.2)
19	F/70		U	-/+	99.0 (5.7)	1.7 (0.3)	98.6 (9.1)
20	F/86				60.1 (1.3)	ND	99.1 (7.33)
21	F/84	A/I	U	ND	37.0 (0.9)	6.0 (0.4)	96.3 (16.8)
22	F/47	B/II	U	+/+	43.9 (0.9)	21.6 (1.0)	96.8 (4.9)
23	M/48	B/II	U	-/+	94.7 (4.2)	10.3 (0.4)	99.2 (7.0)
24	F/80	B/II	M	-/ND	85.5 (8.8)	17.6 (0.4)	90.0 (2.9)
25	M/68	B/II	U	-/-	40.1 (2.5)	31.1 (0.7)	95.4 (7.1)
26	M/73	B/II	M	- /ND	42.91(0.9)	27 (0.7)	86.1 (4)
27	F/75	C/III	M	-/-	70.1 (2.3)	1.46 (0.3)	97.7 (4.9)
28	M/65	B/II	U	-/-	40.1 (0.9)	20.6 (0.7)	99.7 (22.2)
29	F/58	B/II	M	+/-	90.5 (2.4)	2.9 (0.3)	97.7 (6.4)
30	M/80	C/III	ND	+/+	93.2 (2.7)	7.3 (0.3)	99.8 (25.1)
31	F/67	A/0	U	-/+	36.9 (0.8)	2 (1.4)	93.7 (4.8)
32	F/76	B/I	ND	-/-	99.3 (7.8)	1.9 (0.3)	99.5 (16.5)
33	F/80	B/II	ND	+/ND	99.6 (8.8)	6.0 (1.5)	99.7 (16.1)
34	M/66	C/IV	U	-/ND	98.1 (7.9)	3.2 (1.5)	99.6 (27.5)
35	M/83	A/0	M	-/ND	77.1 (1.6)	32.8 (0.6)	99.8 (25.6)
36	M/44	A/0	M	-/ND	99.7 (2.8)	5.2 (0.4)	99.7 (29.3)
37	F/80	A/I	M	-/ND	33.7 (0.7)	15.5 (1.5)	93.5 (7.8)
38	F/82	A/I	M	-/ND	77.6 (1.7)	7.1 (1.8)	97.2 (8.0)
39	M/76	B/II	U	+/ND	40.0 (0.7)	12.3 (0.4)	99.3 (14.3)

[a] Clinical staging system according to Rai and Binet;[1] [b]The mutated (M) or unmutated (U) Ig status is a prognostic marker in CLL;[1] [c]Values represent the percentage of positive cells and, in parenthesis, mean fluoresce intensity; ND, not determined

Supplementary Table S4. Oligonucleotide sequences used in the qPCR analyses

Gene name		Oligonucleotide sequence
ADAM23	*sense*	5′-GCACAGGCTGGGGATTTA- 3′
	antisense	5′-CAGAATCCAACAGTGCAAGG-3′
CCR8	*sense*	5′-TGCCTCCTGTTTGTATTCAGTCT-3′
	antisense	5′-CAGACCACAAGGACCAGGAT-3′
CD226	*sense*	5′-TGCTCTCTTTACACTTACCCACAG-3′
	antisense	5′-GCACAGCTGCCTCAAAACTA-3′
CD274	*sense*	5′-GGCATCCAAGATACAAACTCAA-3′
	antisense	5′-CAGAAGTTCCAATGCTGGATTA-3′
CD52	*sense*	5′-CCTCTTCCTCCTACTCACCATC-3′
	antisense	5′-CTGGTGTCGTTTTGTCCTGA-3′
CD99	*sense*	5′-GCTTCAAAGAAAATGCAGAACA-3′
	antisense	5′-ATTTCTCTAAAAGAGTACGCTGAACA-3′
CD99-I	*sense*	5′-AAAGAAAATGCAGAACAAGGGGA-3′
	antisense	5′-AACAAAGAATCCGCCGTGAA-3′
CD99-II	*sense*	5′-GCTTACCAGAAAAAGAAGCTATGC-3′
	antisense	5′-CACCTCCCCTTGTTCCCTA -3′
CXCR3	*sense*	5′-CCATGGTCCTTGAGGTGAG-3′
	antisense	5′-TCCATAGTCATAGGAAGAGCTGAA-3′
DDHA1	*sense*	5′-CTTCCGGACTGCGTCTTC-3′
	antisense	5′-TGCTTCTTTCATCATGTCAACC-3′
IL10	*sense*	5′-TGGGGGAGAACCTGAAGAC-3′
	antisense	5′-CCTTGCTCTTGTTTTCACAGG-3′
ITGA4	*sense*	5′- GATGAAAATGAGCCTGAAA-3′
	antisense	5′-GCCATACTATTGCCAGTGT-3′
LPP	*sense*	5′-TTCACCTGCGTGATGTGC-3′
	antisense	5′-GCGGGGCAAATTTCTTGT-3′
LRRC16A	*sense*	5′-CGTAGAACGGTCGGATGG-3′
	antisense	5′-CTGGTGTTCCCAAACCAAAG-3′
MAP7D3	*sense*	5′-AACCTGTTTCTCCTCATTTGGAT-3′
	antisense	5′-TGAGTTATCCAGAGTGGAAGATGTAT-3′
MMP9	*sense*	5′-GAACCAATCTCACCGACAGG-3′
	antisense	5′-GCCACCCGAGTGTAACCATA-3′
STAP1	*sense*	5′-TGAGGCCTGGTAGTGACAGTAG-3′
	antisense	5′-AGTGCTTGATTCTTGGAATGTCT-3′
TBP	*sense*	5′-CGGCTGTTTAACTTCGCTTC-3′
	antisense	5′-CACACGCCAAGAAACAGTGA-3′

Supplementary Table S5. siRNAs sequences used in this study

siRNA	Oligonucleotide sequence
MMP-9_1 siRNA	5'-CAUCACCUAUUGGAUCCAAdTdT-3'
MMP-9_2 siRNA	5'-AUUGUAUGCGAUCGCAGACdTdT-3'
$CD99_1$	5'-GCCAGCUGUUCAGCGUACUdTdT-3'
$CD99_2$	5'- CCAGAATCTTGGCTGTTTA dTdT-3'
$\alpha4_1$ siRNA	5'-CUGAAACGUGCAUGGUGGAdTdT-3'
$\alpha4_2$ siRNA	5'-GAACUUAACUUUCCAUGUUdTdT-3'
Control siRNA	5'- AUUGUAUGCGAUCGCAGACdTdT-3'

Supplementary Table S6. Antibodies and Reagents

Antibody	Source	Host Species	Usage
Rabbit isotype control	Immunostep (Salamanca, Spain)	Rabbit	FACS
Mouse isotype control	BD Pharmigen (Franklin Lakes, NJ, USA)	Mouse	FACS
MMP-9 (sc-6841R)	Santa Cruz Biotech. (Santa Cruz, CA, USA)	Rabbit	WB, FACS
CD52 (sc-51560)	Santa Cruz Biotechnology	Mouse	WB, FACS
CD226 (sc-53581)	Santa Cruz Biotechnology	Mouse	WB, FACS
CD99 (#1850, clone DN16)	Bio-Rad (Hercules, CA, USA)	Mouse	WB, FACS
CD99 (#sc-53148, clone 12E7)	Santa Cruz Biotechnology	Mouse	WB
CD274 (#14-5983)	Affymetrix eBioscience (San Diego, CA, USA)	Mouse	WB, FACS
CXCR3 (#353718)	BioLegend (San Diego, CA, USA)	Mouse	WB, FACS
CD38 (16BDH)	Dr. F. Sánchez-Madrid (Madrid, Spain)	Mouse	FACS
α4 integrin (HP2/1)	Dr. F. Sánchez-Madrid	Mouse	FACS
Phospho-p65 (S536, #3033)	Cell Signalling Tech. (Danvers, MA, USA)	Rabbit	WB
Phospho-Sp1 (T453, #37707)	Abcam (Cambridge, UK)	Rabbit	WB
Vinculin (#V9131)	Sigma-Aldrich (St. Louis, MO, USA)	Mouse	WB
Alexa 488-anti-mouse Igs	Molecular Probes (Eugene, OR, USA)	Goat	FACS
Alexa 647-anti-mouse Igs	Molecular Probes	Goat	FACS
HRP-anti-mouse Igs	DAKO Corporation (Hamburg, Germany)	Goat	WB
HRP-anti-rabbit Igs	DAKO Corporation	Goat	WB

Reagent	Source
TNF-α	R&D Systems (Minneapolis, MN, USA)
Recombinant human VCAM-1/CD106 Fc chimera	R&D Systems
CCL21	Peprotech EC (London, UK)
Fibronectin FN-H89 fragment (contains the CS1 ligand for $\alpha4\beta1$ integrin)	Prepared as described[2]
Mithramycin	Sigma-Aldrich
NFκB inhibitor	Calbiochem (Darmstadt, Germany)

Supplementary Methods

Gene expression analysis by microarrays

$3x10^6$ MEC-1 cell transfectants were cultured in IMDM/0.1% FBS for 2 h and total RNA extracted and purified using the Allprep® DNA/RNA/Protein kit (Qiagen, Hilden, Germany). Double-stranded cDNA and biotinylated cRNA were synthesized from 200 ng total RNA using the Ambion® WT Expression Kit (Thermo Fisher Scientific, MA, USA) as described.[3] Biotinlabeled cRNA was fragmented and hybridized to a GeneChip® Human Gene 1.0 ST Array (Affymetrix, Santa Clara, CA). Expression values were normalized and summarized using the Robust Multi-Array Average algorithm.[4,5] Differential expression analyses in MMP-9-cells versus Mock-cells were conducted by the Significance Analysis of Microarray method,[6] setting the false discovery rate to 0.1. Genes with significantly different expression and ≥ 2-fold change were selected and the resulting group functionally annotated using the BP_FAT category of Gene Ontology (GO) and the Database for Annotation, Visualization and Integrated Discovery (DAVID, National Institute of Allergy and Infectious Diseases) v6.7. GO BP_FAT terms with an associated p value of ≤ 0.05 were considered significantly enriched. Heat maps representing gene expression profiles were obtained with TIGR Multiexperiment Viewer v4.9 (TM4 Software Suite, Dana-Farber Cancer Institute, Boston, MA). The complete gene expression data sets have been deposited and are available online at the Gene Expression Omnibus repository (GEO ID: GSE78174).

Quantitative PCR (qPCR)

Total RNA from $3x10^6$ MEC-1 transfectants or $5x10^6$ primary CLL cells, preincubated or not with 110 nM recombinant MMP-9 or MMP-9MutE for 24 h, was isolated using with TRI Reagent (Sigma-Aldrich) or the Allprep® DNA/RNA/Protein kit (Qiagen), following the manufacturer's protocol, 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 Supplementary Table S4. 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.

Flow cytometry analyses

$1.5x10^5$ MEC-1 transfectants or primary CLL cells, treated or not with 110 nM proMMP-9 or proMMP-9MutE for 24 or 48 h, 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 647 (MEC-1 cells) or Alexa 488 (primary CLL cells) labeled secondary Abs. Samples were analyzed on a Cytomics FC500 or a Coulter Epics XL flow cytometer (Beckman Coulter, Fullerton, CA).

RNA interference experiments

The siRNA sequences targeting the various human genes studied and the siRNA control (Supplementary Table S5) were custom-made by Sigma-Aldrich (St. Louis, MO, USA). $15x10^6$ MEC-1 cells or primary CLL cells were nucleofected with 6 μM

siRNAs using solution V and program T-01 (MEC-1 cells) or Human B cell solution and program U-15 (primary CLL cells) (Amaxa, Cologne, Germany), and assayed 24 h or 48 h after transfection. Transfection efficiency was monitored by qPCR, flow cytometry and/or Western blotting.

Lentiviral production and infection

For CD99 expression, lentiviral particles containing the pLenti-C-myC-DDK vector alone (control) or including the CD99 gene were purchased from Origene (Rockville, MD, USA). $2x10^6$ MMP-9-cells were seeded in 24 well plates in 1 ml IMDM/10% FBS containing 5 µg/ml polybrene (Fluka, Steimheim, Switzerland). Cells were incubated with CD99-encoding lentiviral particles or with control particles at a multiplicity of infection of 10. After 24 h, inocula were replaced by fresh medium, and cells further incubated for 48 h. CD99 expression was analyzed by qPCR and flow cytometry.

Western blotting

$2\text{-}5x10^6$ MEC-1 transfectants or $5\text{-}10x10^6$ 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 in the lysates 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.[7] Protein load was corrected using vinculin as internal standard.

Statistical analyses

Normal distribution of the data was confirmed by the Kolmogorov and Smirnov normality test. 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 the GraphPad InStat v3.06 software (GraphPad Software, San Diego, CA, USA). All values are expressed as means ± standard deviation, except for the qPCR and functional assays, in which standard error is shown.

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