Gutiérrez-González A, Aguilera-Montilla N, Ugarte-Berzal E, et al., – Supplemental Data

1. Supplemental methods

Antibodies and reagents

Monoclonal antibodies (mAbs) HP2/1 (anti-α4 integrin subunit, function blocking), HP1/7 (anti-α4 integrin subunit, non-blocking), TS2/16 (anti-β1 integrin subunit), and 16BDH (anti-CD38) were obtained from Dr. F. Sánchez-Madrid (Hospital de la Princesa, Madrid, Spain) and have been previously characterized.[1-4] mAb ALC (anti-α4 integrin subunit, function blocking) was obtained from Dr. Angel Corbí (Centro de Investigaciones Biológicas, Madrid).[5] mAb Act-1 (anti-α4β7 integrin) was provided by Dr. A.I. Lazarovits (University of Western Ontario, Canada)[6]. mAb P1B5 (anti-α3 integrin subunit, function blocking) was from Merck KGaA (Darmstadt, Germany). HP2/1, TS2/16, Act-1, and P1B5 mAbs were also used for flow cytometry. Anti-CD14 mAb and isotype control purified mouse IgG1 were from BD Pharmingen (BD Biosciences, Franklin Lakes, NJ, USA). Rabbit polyclonal (RpAb) anti-VEGFR2 (sc-504, used for immunoprecipitation) and anti-VEGFR2 mAb (sc-393163, used for flow cytometry) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Goat anti-VEGFR2 (AF357, function blocking) was from R&D Systems (Minneapolis, MN, USA). RpAb anti-VEGF (used for tissue staining) was from Antibodies-online GmbH (Aachen, Germany). Alexa Fluor 488 mAb to VEGFR2 and Alexa Fluor 488 mouse IgG1 isotype control were from BioLegend (San Diego, CA, USA). Alexa Fluor 568 goat anti-rabbit Igs was from Invitrogen (Carlsbad, CA, USA). Alexa Fluor 488 goat anti-

mouse Igs was from Jackson ImmunoResearch (Cambridgeshire, UK). RbmAb anti-phospho-VEGFR2 (Tyr1175) and RpAb anti-phospho-Akt (T308) were from Cell Signaling Technology (Danvers, MA, USA). mAb anti-phospho-FAK Y397 was from BD Biosciences and mAb anti-vinculin (V9131) was from Sigma-Aldrich (Saint Louis, MI, USA). VEGFR2 kinase inhibitor I was from Calbiochem (Darmstadt, Germany). VEGF165 (#100-20) was from PeproTech (London, UK). The fibronectin fragment FN-H89, containing the CS1 ligand for $\alpha 4\beta 1$ integrin, was prepared as we previously described.[4] Fibronectin-derived synthetic peptides CS1 (DELPQLVTLPHPNLHGPEILDVPST) and CS3 (IQLPGTSGQQPSVGQQMIFEEHGFR, control peptide) were synthesized on an automated multiple peptide synthesizer (AMS 422, ABIMED Analysen-Technik GmbH, Langenfeld, Germany).

Quantitative PCR

Total RNA isolation and cDNA amplification were performed as described.[7] Quantitative PCR (qPCR) was performed using iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA) and the following primers, custom-made by Sigma-Aldrich: VEGFR2: forward: 5´-GCTCAAGACAGGAAGACCAAG-3´, reverse: 5´-GGTGCCACACGCTCTAGG-3; $\alpha 4$ integrin: forward: 5´-ACCGCTCCTACAGCAAGC-3¨, reverse: 5´-CGCGTTCATGTCGTAATAGTTG-3´. Determinations were performed in triplicate and the results were normalized according to the expression levels of TBP and expressed using the ΔΔCT method for quantization.

Immunoprecipitation and Western blotting

For immunoprecipitation, 15 x 10^6 cells were cultured in medium-0.1% FCS for 2 h and treated or not with 50 ng/ml VEGF for 15 min. Cells were lysed in 10 mM Tris pH 7.5, 150 mM NaCl, 0.5% NP-40 and 1 mM EDTA (lysis buffer). Lysates were pre-cleared by incubating (2 h, 4ºC) with 25 µl protein A-Sepharose or protein G-Sepharose (GE Healthcare, Uppsala, Sweden), depending on whether pAbs or mAbs were used for immunoprecipitation. Pre-cleared lysates were incubated (16 h, 4ºC) with 4 µg of control or appropriate Abs and mixed with 35 µl protein A-Sepharose (pAbs) or protein G-Sepharose (mAbs) for 2 h at 4ºC in rotation. Pellets containing the immune complexes were washed with lysis buffer and proteins extracted with Laemmli buffer by boiling for 10 min. Western blotting analyses of whole cell lysates and immunoprecipitates were performed as we previously reported.[8]

Flow cytometry and Immunofluoresce assays

For VEGFR2 determination, 2 x 10^5 MEC-1 or primary CLL cells, treated or not with 50 ng/ml soluble VEGF for 20 min, were fixed with 2-4% paraformaldehyde (10 min, 4ºC) and incubated (30 min, 4ºC) in 100 µl PBS/1%BSA with or without anti-VEGFR2 mAb sc-393163. Cells were washed, incubated with Alexa 488 labeled secondary antibodies (30 min, 4ºC) and analyzed on a Coulter Epics XL flow cytometer (Beckman Coulter, Fullerton, CA). Other cell surface proteins were determined as above, except that the fixation step was omitted. For inhibition experiments, cells were first treated with appropriate blocking or control

antibodies (30 min, 4ºC) prior to their incubation with VEGF. VEGFR2 in this case was detected with an Alexa-fluor 488-anti-VEGFR2 antibody.

For immunofluorescence analyses, glass coverslips were coated with 10 µg/ml poly-L-lysine or 5 µg/ml VEGF for 2 h at 37°C, washed, and blocked with 1% BSA for 30 min. CLL cells were added to the coverslips and incubated at 37°C for 1 h. Cells were fixed, permeabilized with PBS, 0.2% Triton X-100, and incubated (1 h, RT) with appropriate primary Abs. Cells were blocked with human IgGs (30 min, RT), washed and incubated (15 min, RT) with Alexa 488- or Alexa 568-labeled secondary Abs. Images were acquired using a Leica TCS-SP2-AOBS-UV microscope with ACS APO 63x/NA 1.30 oil immersion objective. The "Dye-Separation Leica software" was used for colocalization studies. Image pixels were depicted as dot-plot representations, where X and Y correspond to the fluorescence intensity value of each fluorescence channel per pixel. A co-localization region was assigned to pixels displaying high levels of fluorescence for the two analyzed colors. Pearsons coefficient was used to quantify the degree of colocalization between fluorophores.

Immunohistochemistry analyses of VEGF

Paraffin-embedded slides were de-paraffinized, rehydrated and unmasked by steaming in 10 mM sodium citrate buffer (pH 6.0) for 20 min. Slides were blocked with 5 µg/ml human IgG solved in blocking serum-free medium (Dako Denmark A/S, Glostrup, Denmark) for 30 min and incubated overnight at 4°C with 5 µg/ml primary Abs. Slides were washed with PBS-Tween 0.05% and incubated for 1h at RT with Alexa Fluor 488- or Alexa Fluor 568-secondary Abs. After washing,

samples were mounted with Fluorescence mounting medium (Dako) and imaged with a Leica SPE confocal microscope using the glycerol-immersion ACS APO 20x/NA 0.60 objective.

Cell adhesion assays

96-well plates were coated with several concentrations of subtrates, followed by blocking with 0.1% BSA. Cells ($1.5x10^5$/well) 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, 0.1% BSA, 1 mM Mn^{2+}, and added to the coated wells. After 60 min at 37°C, attached cells were lysed with PBS, 0.1% SDS and quantified using a fluorescence analyzer (BMG Labtech, Offenburg, Germany). For inhibition experiments, cells were incubated (30 min, 37°C) with appropriate Abs (10 µg/ml) or peptides (500 µg/ml) before adding to the wells and adhesion was measured as above.

Cell viability assays

Cell viability was measured by the Cell Counting Kit-8 (CCK-8) method (Sigma-Aldrich). 2 x 10^5 primary CLL cells were serum starved for 2 h and incubated (30 min, 37°C) with blocking or control Abs (10 µg/ml) or VEGFR2 kinase inhibitor I (5 µM). Cells were incubated on 96-well plates coated with 5 µg/ml VEGF or 0.1% BSA for 48 h and 5 µL of CCK-8 reagent were added for the last 4 h. The absorbance at 450 nm was determined using a Multiskan Bichromatic microplate reader (Labsystems, Helsinki, Finland).

VEGF-induced intracellular signaling

5 x 10^6 CLL cells were treated or not with VEGFR2 kinase inhibitor or appropriate antibodies for 30 min at 37°C, and added to 24-well plates previously coated with 0.1% BSA or 5 μg/ml VEGF. After 15 min at 37°C, cells were collected, lysed in lysis buffer, and analyzed by Western blotting.

Statistical analyses

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

References

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2. Supplemental Tables

Table S1. Clinical characteristics of CLL patients

Patient	Sex/Age	Stage	Ig Status	CD38	α4[a]	β1[a]	α3[a]	VEGFR2[a]
1	M/75	B/II	U	+	75.7	73.9	74.6	33
2	M/79	A/I	U	-	62.8	91.8	91.8	17
3	M/69	C/IV		-	37.0	72.7	16.7	39
4	F/55	C/IV	M	+	94.1	95	89.3	38
5	F/88	B/II	U	+	61.0	85.8	21.1	64.6
6	M/70	B/II	U	-	16.6	24.7	6.5	76.1
7	M/58	B/II	U	-	27.8	25.1	37.5	13.5
8	F/84	C/III	U	+	23.0	43.8	36.7	23
9	F/46	B/II	U	+	78.3	95.8	49	+[b]
10	F/59	B/II	U	-	36.2	20.6	21.8	+[b]
11	F/54	A/0	U	+	92.5	82.5	62.6	+[b]
12	F/88	B/II	U	+	62.1	49.8	7.8	+[b,c]
13	F/63	B/I	M	-	90.5	68.1	5.3	26.9
14	M/83	A/I	M	-	83.8	60.8	1.3	39.5
15	M/59	B/II	U	-	59.5	18.7	22.7	31.7
16	M/66	B/II	U	-	45.2	25.3	30.7	15.3
17	F/83	A/0	M	-	77.6	82.7	77.0	38.1
18	M/77			-	79.3	84.9	Nd	42.9
19	M/80	B/II	U	+	97.1	94.6	Nd	68.5
20	M/66	B/II	U	-	66.7	68.3	Nd	74.2
21	M/70	B/II	M	-	54.8	49.2	Nd	30.7
22	M/78	B/II	M	-	30.6	28.1	Nd	68.7
23	F/84	C/III	U	-	77.6	71.6	Nd	24.7
24	F/88	B/II	U	-	17.6	20.5	1.2	64.2
25	M/80	B/II	U	-	19.6	12.3	21.5	25.7
26	F/67	A/0	U	-	36.9	Nd	Nd	14.4
27	M/80	C/III		+	93.2	90.4	Nd	33.4
28	F/76	B/I		-	99.2	94.8	Nd	13.1
29	F/58	B/II	M	+	90.5	46.8	Nd	26.2
30	M/63	B/II	M	-	47.9	28.1	3.4	22.4

31	M/55	A/0	Nd	-	56.4	75.8	73.3	12.9
32	M/67	B/II	M	-	39.8	29.1	34.2	10.4
33	F/65	A/I	M	-	30.0	18.0	51.9	31.0
34	M/58	C/IV	M	+	63.5	38.6	13.2	48.0
35	F/65	C/III			98.2	95.4	89.6	59.5
36	F/65				60.8	88.2	69.2	77.7
37	F/49	B/II	M	-	23.0	Nd	9.8	74.2
38	F/83	C/III	M	-	33.6	38.2	17.0	37.4

[a] Values represent the percentage of positive cells. U, unmutated. M, mutated. Nd, not determined. [b]Samples P9, P10, P11 and P12 expressed functional VEGFR2, as shown in Figures 1A,B (P9), 1E (P11), 2B (P12) and 2C (P10). [c]Expression of VEGFR2 in P12 was also demonstrated by qPCR analyses (not shown). Expression of α4β7 integrin was analyzed by flow cytometry in samples P19, P20, P21, P22, and P23, rendering negative values (<5%) in all cases.

Table S2. VEGFR2 expression in normal B cells

Sample[a]	VEGFR2[b]
1	11.7
2	46.3
3	46.2
4	32.2
5	32.2
6	26.3

[a]B lymphocytes were purified from buffy coats of six different donors.
[b]Values represent the percentage of positive cells.

3. Supplemental Figures

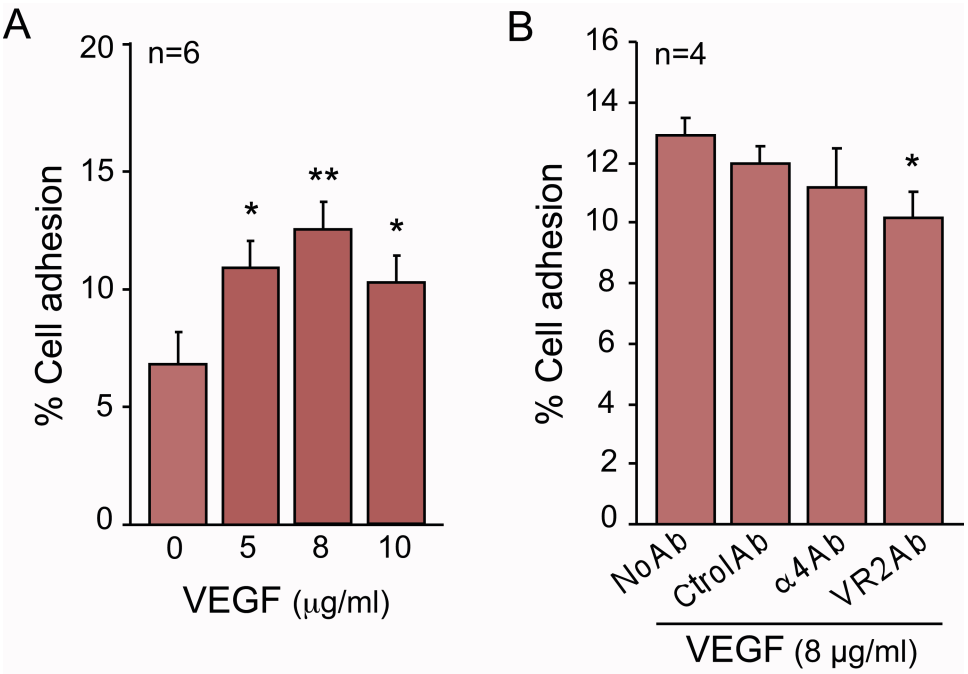

Figure S1. Adhesion of normal B lymphocytes to VEGF. (A) BCECF-AM-labeled normal B lymphocytes (six different donors) were added to wells coated with the indicated concentration of VEGF. After 60 min at 37°C, attached cells were quantified using a fluorescence analyzer. (B) Normal B lymphocytes (four different donors), with or without previous incubation with the indicated antibodies, were added to wells coated with 8 μg/ml VEGF and adhesion was quantified as explained. Average values ± SEM are shown. *, $p < 0.05$; **, $p \leq 0.01$.

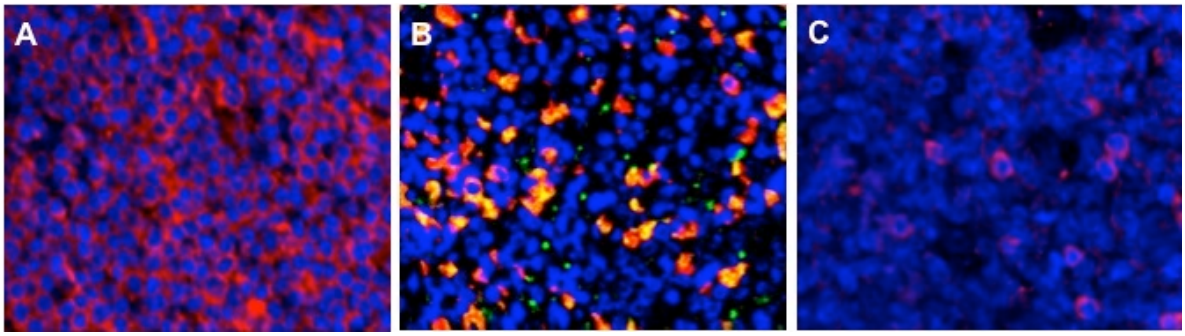

Figure S2. CLL tissues express VEGF. The presence of VEGF (red) was analyzed in four lymph node and two bone marrow samples from CLL patients and representative cases are shown. (A) CLL lymph node stained with anti-VEGF antibody (red). (B) CLL bone marrow stained with anti-VEGF (red) and anti-CD19 (green) antibodies. Co-localization of VEGF and CD19 was observed in many cells, strongly suggesting that CLL cells infiltrating the bone marrow were positive for VEGF. (C) VEGF expression in the follicular region of a representative normal lymph node (four samples analyzed). Cell nuclei were stained with DAPI.

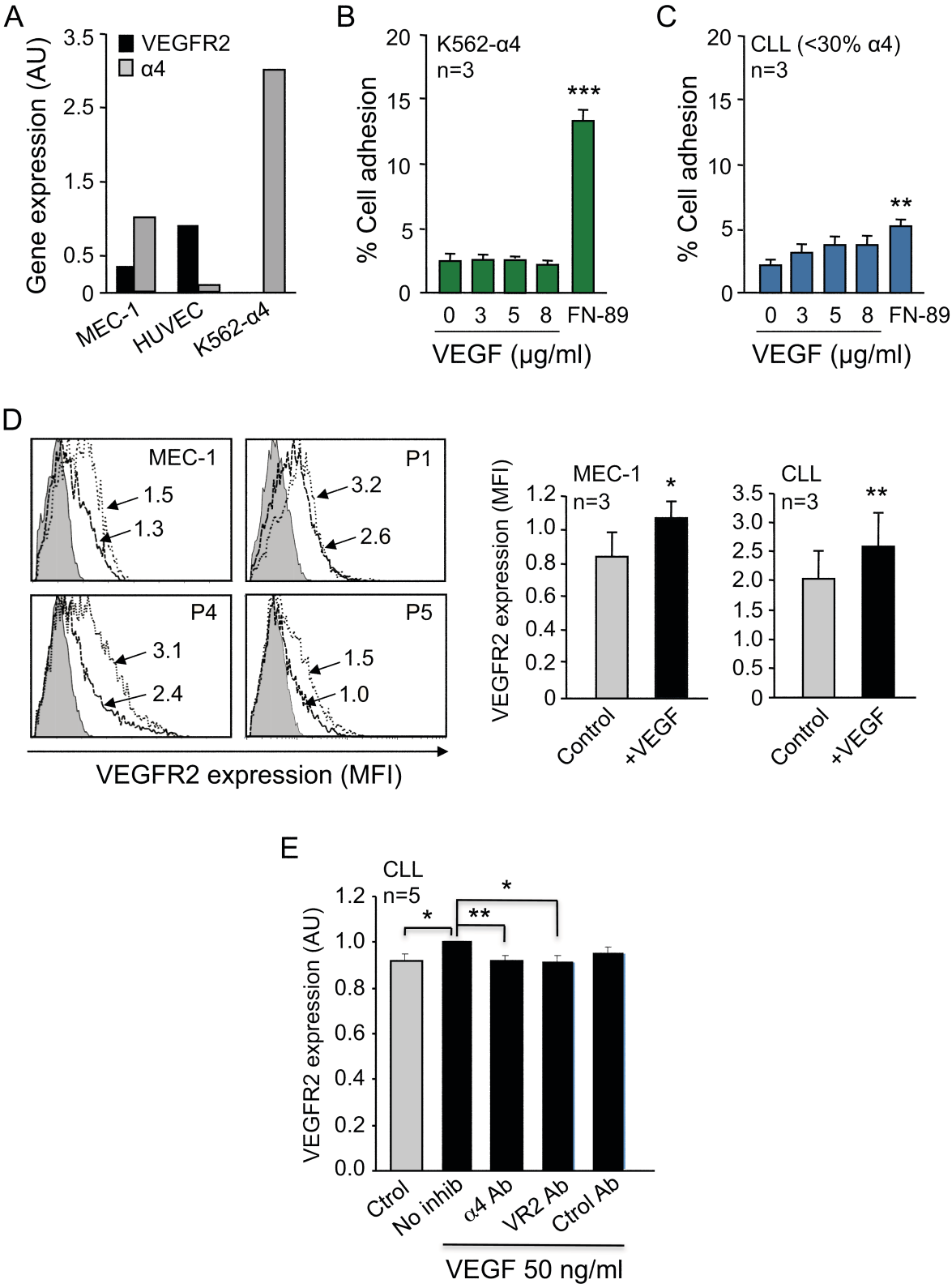
A
Gene expression (AU)
VEGFR2
α4
MEC-1
HUVEC
K562-α4
B
K562-α4
n=3
% Cell adhesion
***
0 3 5 8 FN-89
VEGF (μg/ml)
C
CLL (<30% α4)
n=3
% Cell adhesion
**
0 3 5 8 FN-89
VEGF (μg/ml)
D
MEC-1
1.5
1.3
P1
3.2
2.6
P4
3.1
2.4
P5
1.5
1.0
VEGFR2 expression (MFI)
MEC-1
n=3
VEGFR2 expression (MFI)
Control
+VEGF
CLL
n=3
Control
+VEGF
E
CLL
n=5
VEGFR2 expression (AU)
Ctrol
No inhib
α4 Ab
VR2 Ab
Ctrol Ab
VEGF 50 ng/ml

Figure S3. **CLL cell binding to VEGF involves cooperation between α4β1 integrin and VEGFR2 and results in VEGFR2 redistribution to the cell surface**. (A) qPCR analyses of VEGFR2 (VR2) and α4 integrin gene expression in the indicated cell types. (B) K562-α4 cells were labeled with BCECF-AM and added to wells coated with the indicated concentrations of VEGF or 5 μg/ml FN-H89. After 60 min at 37°C, attached cells were quantified using a fluorescence analyzer. (C) Adhesion of CLL cells with low α4 expression (P6, P7, P8) to the indicated substrata. (D) MEC-1 cells (3 different experiments) and primary CLL cells (patients P1, P4, P5) were stimulated or not with 50 ng/ml VEGF for 20 min and the expression of VEGFR2 was analyzed by flow cytometry. Grey areas: control cells; continuous lines: untreated cells; dotted lines: VEGF-treated cells. Numbers indicate mean fluorescence intensity (MFI). (E) Primary CLL cells (patients P19, P20, P22, P23, P34) were untreated or treated with the indicated antibodies (30 min, 4ºC) and stimulated with VEGF as explained in (D). The expression of VEGFR2 (VR2) was analyzed by flow cytometry. The MFI values of VEGF-stimulated cells, untreated with antibodies, were normalized to 1. Average values ± SEM are shown. *, $p < 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.