CD38/CD31 Interactions Activate Genetic Pathways Leading to Proliferation and Migration in Chronic Lymphocytic Leukemia Cells

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Human CD38 is a pleiotropic glycoprotein belonging to a family of enzymes/receptors involved in the catabolism of extracellular nucleotides. CD38-receptor activities are regulated through binding to the nonsubstrate ligand CD31. CD38 expression above a critical threshold is a negative prognostic marker for chronic lymphocytic leukemia (CLL) patients. Activation of CD38 by means of agonistic monoclonal antibodies or the CD31 ligand induces proliferation and immunoblast differentiation of CLL cells. Here we define the genetic signature that follows long-term *in vitro* interactions between CD38$^+$ CLL lymphocytes and CD31$^+$ cells. The emerging profile confirms that the CD31/CD38 axis activates genetic programs relevant for proliferative responses. It also indicates a contribution of this pathway to the processes mediating migration and homing. These results further support the notion that the CD31/CD38 axis is part of a network of accessory signals that modify the microenvironment, favoring localization of leukemic cells to growth-permissive sites.

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INTRODUCTION

Human CD38 is a pleiotropic glycoprotein belonging to a complex family of enzymes of the cell surface involved in the catabolism of extracellular nucleotides (1). During evolution, CD38 acquired the ability to mediate cell–cell interactions, acting as a receptor and binding the nonsubstrate ligand CD31. CD31/CD38 interactions drive activation and proliferation of distinct lymphocyte populations (2).

Along with the absence of mutations in the immunoglobulin variable region heavy chain gene (*IgVH*) (3) and expression of the cytoplasmic kinase ZAP-70 (4), CD38 expression above a critical threshold is a reliable negative prognostic marker for chronic lymphocytic leukemia (CLL) patients (5). The working hypothesis of our group was that CD38 is not merely a marker in CLL, but is also a cell surface receptor and adhesion molecule directly involved in the delivery of growth signals (6). Several pieces of evidence support this view. First, CD38 expression is higher within the bone marrow and the lymph nodes, where the proliferative core of the disease resides (7). Moreover, within each CLL clone, cells expressing CD38 are enriched in expression of Ki-67, suggesting that CD38$^+$ cells are a cycling subset (8). The CD38$^+$ subset is also characterized by a specific genetic profile, showing upregulation of proliferation/survival pathways (9).

We showed that activation of CD38 induces proliferation and immunoblast differentiation of CLL cells (10). These signals are regulated by physical localization of the CD38 receptor within lipid microdomains and act in synergy with the B-cell receptor/CD19 complex (11). The initial observations were obtained by use of agonistic monoclonal antibodies and then extended to a model closely resembling the physiological environment in which CLL cells grow and expand. Indeed, CD38$^+$ CLL cells can bind to murine fibroblasts transfected with the CD31 ligand, with resulting increased growth and survival (12). *In vivo*, several CD31$^+$ cells can be found in lymphoid organs, often in close contact with CD38$^+$ CLL cells (13).

This work was undertaken to obtain a genome-wide signature of the transcriptional events that follow activation of the CD31/CD38 axis. It also provides some answers to the issues raised by a recent report, in which the relevance of CD31/CD38 interactions in the context of CLL

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was called into question (14). The emerging genetic profile indicates that CD31/CD38 interactions are indeed followed by activation of genetic programs relevant not only for proliferative responses, as could be anticipated on the basis of *in vitro* experiments, but also for migration and homing. This latter point deserves further attention and suggests a potential role for CD38 in directing CLL cells to specific microenvironments.

MATERIALS AND METHODS

Patients and Cells

We obtained 10 samples from CLL patients presenting with typical morphology and immunophenotype after we obtained written informed consent in accordance with institutional guidelines and the Declaration of Helsinki. Analyses included CD38 and ZAP-70 stainings (cutoff positivity values of ≥20% and ≥10%, respectively [15]), IgVH mutational status (unmutated if ≥98% homology to the germline gene) and fluorescence *in situ* hybridization (FISH) for chromosomes 11, 12, 13 and 17 (Table 1).

B cells were purified from peripheral blood mononuclear cells by negative selection (15).

Table 1. Clinical and biological features of the CLL patients.

Patient no.	Age, years	Sex	Stage[a]	YD	TX	IgVH, %[b]	CD38, %	ZAP-70, %	FISH
1	60	F	0/A	10	T	100	54	2	11q–
2	65	F	2/A	7	T	100	35	12	11q–,13q–
3	72	F	0/A	7	NT	—	70	11	+12,17p–
4	47	M	2/A	21	T	91.2	75	0	ND
5	57	M	4/C	7	T	97	79	9	ND
6	83	M	2/A	5	T	99.7	66	23	+12
7	75	F	1/B	6	NT	99	42	12	17p–
8	61	M	4/A	8	NT	99.3	78	21	ND
9	48	M	2/B	8	T	99.1	34	35	13q–
10	67	M	2/A	13	T	99	58	14	+12

[a]Stage defined according to Rai and Binet diagnostic criteria. YD, years since diagnosis; TX, therapy; NT, untreated; —, missing data; ND, none detected.
[b]Shown as percentage similarity to the closest germline gene.

Culture Conditions

Mouse L-cells transfected with human *CD31* (L-CD31$^+$) or with the empty plasmid (L-mock) were obtained as described (16). Freshly purified CLL cells (1.5×10^6) were plated on mitomycin C–inactivated (0.5 mg/mL, 20 min at 37°C) L-CD31$^+$ or L-mock fibroblasts (2×10^5/well in a 24-well plate) for 5 d.

Gene Profiling

Data collection. RNA was obtained from freshly purified CLL cells (basal) or after culture on L-CD31$^+$ (L-CD31$^+$ profile) or L-mock fibroblasts (L-mock profile). Amplified and labeled specimens from each sample were combined with the Human Universal Reference Total RNA (Clontech, Saint-Germain-en-Laye, France), fragmented and hybridized to oligonucleotide glass arrays representing 41,000 human unique genes and transcripts (Human Whole Genome Oligo Microarray Array, Agilent Technologies, Cernusco, Italy) (15). A dye-swap replicate was performed for each sample.

Data analysis. Data were preprocessed using the Rosetta Resolver SE software (Rosetta Biosoftware, Seattle, WA, USA) (15). Error-weighted Student two-sample paired *t* test with Benjamini and Hochberg *P* value adjustment was applied to obtain differentially expressed sequences in the L-CD31$^+$ versus L-mock, L-CD31$^+$ versus basal and L-mock versus basal class comparisons, after a sequence prefiltering step (average expression ratio *P* value with respect to the Universal Reference less than 0.01 in at least 50% of the patients of each class comparison). Venn diagrams were then created to visualize intersections between class comparison results and to select the sequences of interest.

Pathway analysis. Significant differential expression at the level of a collection of predefined pathways from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database was identified using Pathway-Level Analysis of Gene Expression (PLAGE) (http://dulci.org/pathways/) (17).

Hierarchical clustering. Results were represented using Cluster 3.0 and TreeView (http://rana.lbl.gov/EisenSoftware.htm) and applying hierarchical clustering to the pathway matrix output given by PLAGE.

Web Deposition of Data

Data in this study have been deposited in the Gene Expression Omnibus (GEO) site (http://www.ncbi.nlm.nih.gov/geo), accession number GSE14063.

All supplementary materials are available online at www.molmed.org.

RESULTS AND DISCUSSION

CD38 receptor activities are regulated through binding to CD31, a nonsubstrate ligand surrogated by ligation with agonistic monoclonal antibodies [reviewed in (1)]. *In vitro* experiments showed that these interactions induce marked proliferation and immunoblast transformation of a fraction of the leukemic clone. We asked whether these results would enable us to find a confirmation by use of a different technological approach, also needed because the relevance of the CD31/CD38 axis in CLL has recently been called into question (14). We addressed this issue by obtaining a genome-wide signature of the events taking place after CD31/CD38 interactions. To this aim, CLL cells from 10 CD38$^+$ patients were used immediately (basal profile) or cultured for 5 days in the presence of L-CD31$^+$ transfectants (L-CD31$^+$ profile) or of mock-transfected cells (L-mock profile) used as controls. The

choice of the 5-day time point was based on previous results indicating that proliferation and survival are highest after this incubation time (12). Moreover, this approach rests on physiological considerations in that CD31/CD38 static interactions are likely to take place within lymphoid organs, in which leukemic cells lie in close and prolonged contact with $CD31^+$ residential elements (13).

Sample and reference RNAs were cohybridized to arrays representing 41,000 human unique genes and transcripts. The strategy selected to identify the signature in response to CD31/CD38 interactions relied on identification of genes differentially expressed in the L-$CD31^+$ versus L-mock comparison and subsequent exclusion of genes modulated in the L-mock versus basal comparison. Results are presented in the Venn diagram of Figure 1. Two *P* value thresholds were adopted, one to exclude genes that changed expression simply as a result of coculture (L-mock versus basal, *P* value 0.01), and the other to select sequences with a more significant level of modulation (L-$CD31^+$ versus L-mock and L-$CD31^+$ versus basal, *P* value 0.001) among the genes differentially expressed as a direct consequence of coculture on L-$CD31^+$ transfectants.

The result was the identification of 1645 sequences modulated after CD31/CD38 interactions. The list of genes contained in the pathways of the KEGG with a significant enrichment score (adjusted *P* value < 0.01) is available as Supplementary Table 1. PLAGE of the sequences identified 85 differentially regulated pathways (Figure 2 and Supplementary Table 2). Of these, 14 pathways (16%) are involved in lymphocyte signaling (black arrows in Figure 2), and 12 pathways (14%) are implicated in cell adhesion/movement phenomena (red arrows in Figure 2). Relevant examples of upmodulated genes regulating activation/proliferation pertain to the B-cell receptor, the mitogen-activated protein kinase– and the Toll-like receptor–signaling pathways (Figure 2 and Supplementary Table 2). Specific attention was dedicated

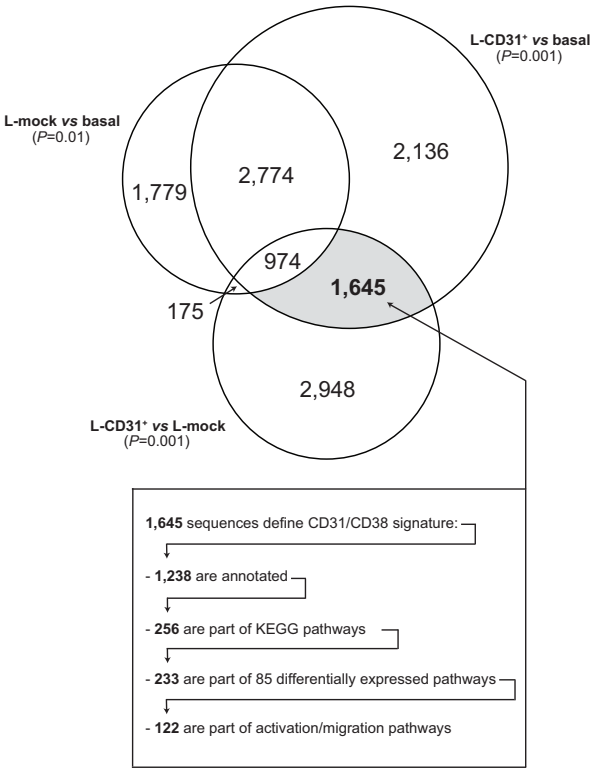

Figure 1. CD38 engagement by the CD31 ligand triggers genetic pathways leading to cell proliferation and movement. Venn diagram showing the strategy selected to identify the signature in response to CD31/CD38 interactions. Results were obtained by applying error-weighted Student two-sample paired *t* test with Benjamini and Hochberg *P* value adjustment to L-$CD31^+$ versus L-mock, L-$CD31^+$ versus basal and L-mock versus basal class comparisons. Circle radii are proportional to the number of transcripts differentially expressed in each condition. The gray area defines the CD31/CD38 signature. A cutoff of 0.01 for the adjusted *P* value was applied to exclude genes that changed expression simply as a result of coculture (L-mock versus basal), whereas a more stringent threshold (adjusted $P < 0.001$) was applied to the other two comparisons. The resulting 1645 sequences are involved in 85 differentially regulated pathways identified by PLAGE. Of these pathways, 30% are implicated in lymphocyte adhesion/movement and/or signaling.

to the panel of 30 apoptosis-regulating genes that Tonino and colleagues found to be unmodulated (14). In our series, 7 of 30 genes were differentially regulated upon CD31/CD38 interactions, including *P19*, *Bax*, *Bcl-RAMBO*, *Bim*, *PUMA*, *Harakiri* and *Mcl-1* (Figure 3). All of these genes, with the exception of *Mcl-1*, are upregulated upon CD31/CD38 interactions, suggesting that deregulation of apoptosis is a component of this signature. The apparent contrast of these results with those of Tonino *et al*. may be attributable to experimental differences (3T3 cells versus L-fibroblasts, 72 versus 120 hours incubation) and/or to characteristics intrinsic to the patients selected.

In the context of the control of cell movement, relevant examples are upmodulation of genes ruling leukocyte transendothelial migration, actin cytoskeleton and focal adhesion on the one hand, and downregulation of genes influencing interactions with the extracellular matrix and coding for cell adhesion molecules on the other. This genetic signature implies that long-term CD31/CD38 interactions modulate the competence of CLL cells to grow and progress in response to microenvironmental signals and condi-

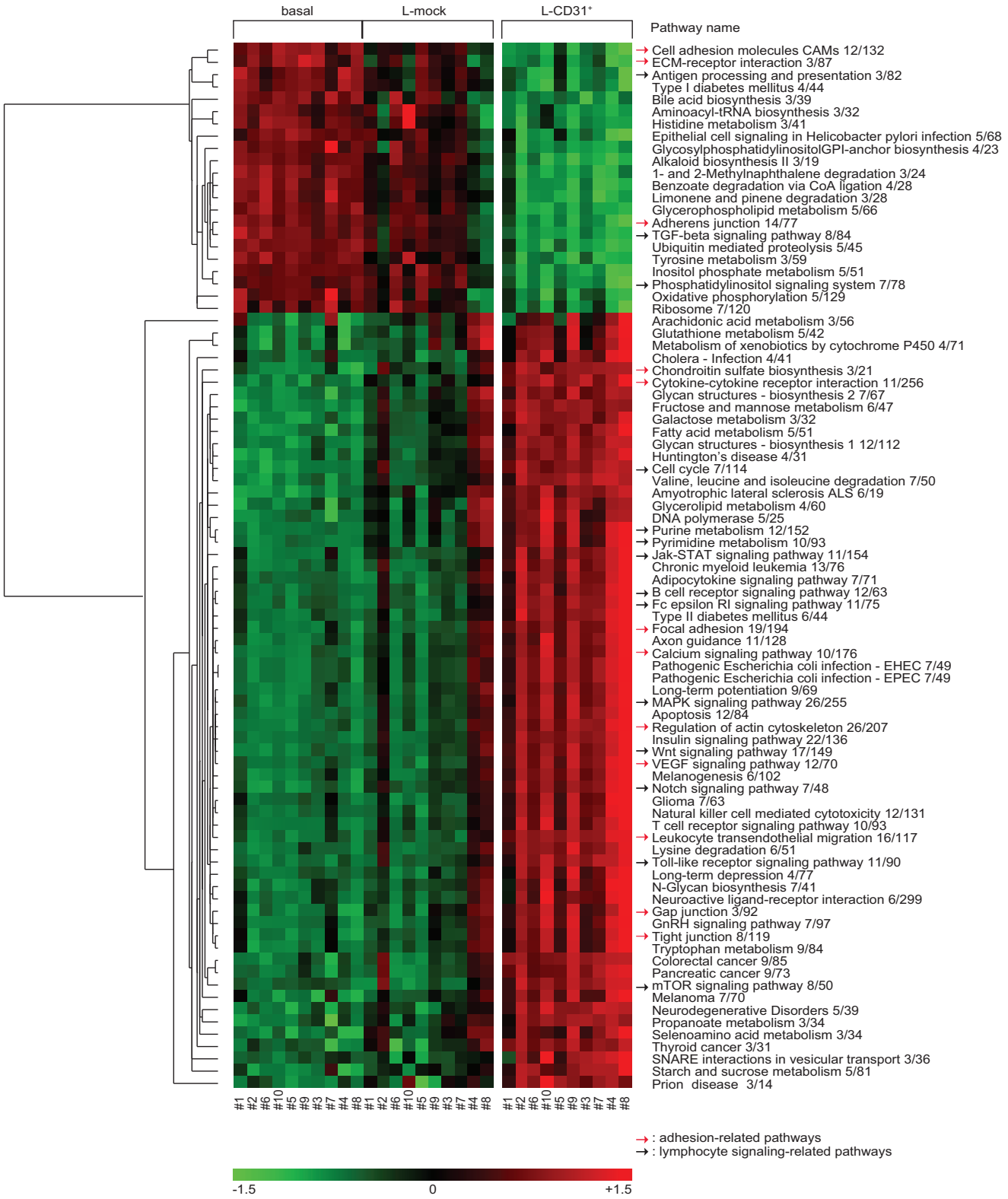

Figure 2. Representation of the expression pattern of 85 pathways differentially expressed as a consequence of CD31/CD38 interactions. Hierarchical clustering was applied to the matrix of the activity levels of the 85 KEGG pathways found by PLAGE analysis to be differentially expressed in the L-CD31$^+$ samples compared with the basal and L-mock ones.

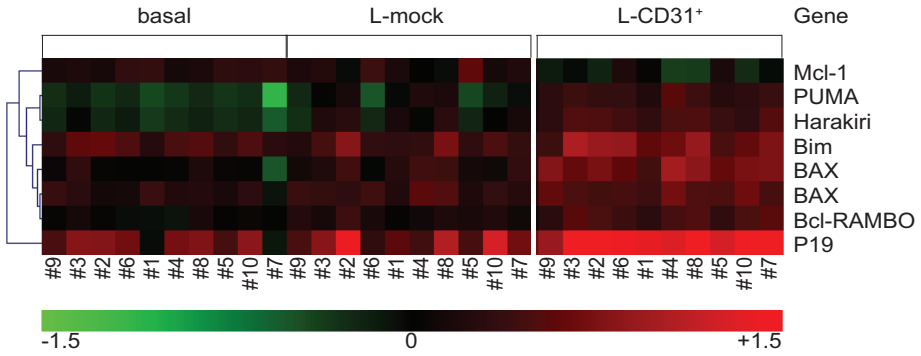

Figure 3. Representation of the seven apoptosis-related genes differentially expressed upon CD31/CD38 engagement. Expression pattern of 7 genes of the 1645 gene signatures that are also part of the 30 apoptosis-regulating genes reported in (14). Red squares refer to upregulation in the sample with respect to the Universal Reference, and green squares to downregulation. Expression ratios are represented in a log 10 scale. All the genes except *Mcl-1* increased their expression as a consequence of CD31/CD38 interactions. Two different Agilent probes specific for *Bax* are overexpressed in the L-CD31$^+$ condition.

tions. Independent immunohistochemical studies on lymph node sections revealed a direct association between the number of endothelial cells (CD31$^+$) and the level of CD38 expression by CLL cells (13), indirectly confirming that CD31/CD38 static interactions occur *in vivo*. Moreover, the percentage of CD38$^+$ CLL cells (8,18) and the density of tumor vessels (19,20) correlate with neoplastic proliferation and disease aggressiveness.

In conclusion, these results provide further support to the hypothesis that CLL maintenance and progression derive not only from the essential drive provided by the antigen but also from a number of accessory signals that modify the microenvironment to favor localization of neoplastic cells to growth-permissive sites. The CD31/CD38 axis is part of this network.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

REFERENCES

1. Malavasi F, *et al*. (2008) Evolution and function of the ADP ribosyl cyclase/CD38 gene family in physiology and pathology. *Physiol. Rev*. 88:841–86.
2. Deaglio S, *et al*. (2000) CD38/CD31, a receptor/ligand system ruling adhesion and signaling in human leukocytes. *Chem. Immunol*. 75:99–120.
3. Damle RN, *et al*. (1999) Ig V gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia. *Blood*. 94:1840–7.
4. Crespo M, *et al*. (2003) ZAP-70 expression as a surrogate for immunoglobulin-variable-region mutations in chronic lymphocytic leukemia. *N. Engl. J. Med*. 348:1764–75.
5. Matrai Z. (2005) CD38 as a prognostic marker in CLL. *Hematology*. 10:39–46.
6. Deaglio S, Vaisitti T, Aydin S, Ferrero E, Malavasi F. (2006) In-tandem insight from basic science combined with clinical research: CD38 as both marker and key component of the pathogenetic network underlying chronic lymphocytic leukemia. *Blood*. 108:1135–44.
7. Jaksic O, *et al*. (2004) CD38 on B-cell chronic lymphocytic leukemia cells has higher expression in lymph nodes than in peripheral blood or bone marrow. *Blood*. 103:1968–9.
8. Damle RN, *et al*. (2007) CD38 expression labels an activated subset within chronic lymphocytic leukemia clones enriched in proliferating B cells. *Blood*. 110:3352–9.
9. Pepper C, *et al*. (2007) Highly purified CD38(+) and CD38(-) sub-clones derived from the same chronic lymphocytic leukemia patient have distinct gene expression signatures despite their monoclonal origin. *Leukemia*. 21:687-96.
10. Deaglio S, *et al*. (2003) CD38 is a signaling molecule in B-cell chronic lymphocytic leukemia cells. *Blood*. 102:2146–2155.
11. Deaglio S, *et al*. (2007) CD38/CD19: a lipid raft dependent signaling complex in human B cells. *Blood*. 109:5390–8.
12. Deaglio S, *et al*. (2005) CD38 and CD100 lead a network of surface receptors relaying positive signals for B-CLL growth and survival. *Blood*. 105:3042–50.
13. Patten PE, *et al*. (2008) CD38 expression in chronic lymphocytic leukemia is regulated by the tumor microenvironment. *Blood*. 111:5173–81.
14. Tonino SH, Spijker R, Luijks DM, van Oers MH, Kater AP. (2008) No convincing evidence for a role of CD31-CD38 interactions in the pathogenesis of chronic lymphocytic leukemia. *Blood*. 112:840–3.
15. Deaglio S, *et al*. (2007) CD38 and ZAP-70 are functionally linked and mark CLL cells with high migratory potential. *Blood*. 110:4012–21.
16. Deaglio S, *et al*. (1998) Human CD38 (ADP-ribosyl cyclase) is a counter-receptor of CD31, an Ig superfamily member. *J. Immunol*. 160:395–402.
17. Tomfohr J, Lu J, Kepler TB. (2005) Pathway level analysis of gene expression using singular value decomposition. *BMC Bioinformatics*. 6:225.
18. Lin TT, *et al*. (2008) Highly purified CD38 subpopulations show no evidence of preferential clonal evolution despite having increased proliferative activity when compared with CD38 subpopulations derived from the same chronic lymphocytic leukaemia patient. *Br J Haematol* 142:595–605.
19. Letilovic T, Vrhovac R, Verstovsek S, Jaksic B, Ferrajoli A. (2006) Role of angiogenesis in chronic lymphocytic leukemia. *Cancer* 107:925–34.
20. Shanafelt TD, Kay NE. (2006) The clinical and biologic importance of neovascularization and angiogenic signaling pathways in chronic lymphocytic leukemia. *Semin. Oncol*. 33:174–85.