THE ROLE OF CD49d IN CHRONIC LYMPHOCYTIC LEUKAEMIA: MICROENVIRONMENTAL INTERACTIONS AND CLINICAL RELEVANCE

***Michele Dal Bo,[1] Erika Tissino,[1] Dania Benedetti,[1] Chiara Caldana,[1] Riccardo Bomben,[1] Giovanni Del Poeta,[2] Gianluca Gaidano,[3] Francesca Maria Rossi,[1] Antonella Zucchetto,[1] Valter Gattei[1]**

1. Clinical and Experimental Onco-Hematology Unit, Centro di Riferimento Oncologico, I.R.C.C.S., Aviano (PN), Italy
2. Division of Hematology, S. Eugenio Hospital and University of Rome Tor Vergata, Rome, Italy
3. Division of Hematology, Department of Clinical and Experimental Medicine, Amedeo Avogadro University of Eastern Piedmont, Novara, Italy
**Correspondence to micheledalbo@gmail.com*

Disclosure: No potential conflict of interest.
Received: 25.03.14 **Accepted:** 20.05.14
Citation: EMJ Hema. 2014;1:80-87.

ABSTRACT

Chronic lymphocytic leukaemia (CLL) is a clinically heterogeneous disease characterised by the accumulation/expansion of a clonal population of neoplastic cells with the morphological appearance of small mature B lymphocytes in blood, bone marrow, and lymphoid organs. Stimulation through the B cell receptor (BCR) plays a prominent role in the selection and expansion of the malignant clone in CLL. On the other hand, other external signals delivered by several cell types including T lymphocytes, macrophages, stromal cells, endothelial cells, and follicular dendritic cells, operating through either direct BCR-independent cell-cell contact or indirect production of paracrine soluble factors, synergistically cooperate in regulating proliferation and survival of CLL cells. In this context, CD49d is known to play a pivotal role in mediating both cell-cell and cell-matrix interactions in CLL-involved tissues, eventually delivering pro-survival signals and protecting CLL cells from drug-induced damages. In the present review, we focused on functional and physical interactions of CD49d with other microenvironmental receptors, including CD38 and BCR, and other specific CD49d-dependent interactions in lymph node and bone marrow microenvironments responsible for growth and survival-supporting signals, eventually influencing CLL prognosis and therapeutic options.

Keywords: CD49d, microenvironment, chronic lymphocytic leukaemia (CLL).

INTRODUCTION

Chronic lymphocytic leukaemia (CLL) represents the most common form of leukaemia in the Western world with an incidence of 3-5 per 100,000 patients. This disease affects mainly elderly people, with a median age at the diagnosis of 70 years, and a little predominance in men.[1,2] CLL diagnosis implies the presence of neoplastic B cells >5000/μL in the peripheral blood; the disease always involves the bone marrow whereas enlargement of lymph nodes, spleen, or liver may be absent. The clinical heterogeneity in this disease is evidenced by the fact that some patients show a refractory disease and die within 2-3 years after the diagnosis, whereas in others the disease has a very indolent course without need of treatment and survival reaching decades. In this context, clinical, genetic, and biological parameters have been introduced to characterise this heterogeneity and to evaluate the prognosis of CLL patients.[2] In particular, specific chromosomal aberrations (i.e. deletion 17p, deletion 11q or trisomy 12), the presence of unmutated immunoglobulin heavy chain (IGHV) genes, as

well as a mutated configuration of the TP53, NOTCH1, SF3B1, and BIRC3 genes, or expression levels for ZAP-70, CD38, and CD49d exceeding the value of an established threshold, have been reported to correlate with a poor clinical outcome in CLL.[2-4]

The biological parameters employed as prognosticators can be molecules involved in the functional interplay of CLL cells with the neighbouring cells which form the tissue microenvironment of CLL cells.[1,2,5] As an example, stimulation through the B cell receptor (BCR) plays a prominent role in the selection and expansion of the malignant clone in CLL, and the prognostic impact of the mutational status of IGHV genes could be considered as a consequence of the relevance of this process in CLL.[1,6] Other external signals delivered by several cell types including T lymphocytes, macrophages, stromal cells, endothelial cells, and follicular dendritic cells (DCs), operating through either direct BCR-independent cell-cell contact or indirect production of paracrine soluble factors, synergistically cooperate in regulating proliferation and survival of CLL cells.[1,7-12]

In this context, in CLL-involved tissues of bone marrow and lymph nodes, CD49d is known to operate as one of the master molecules mediating both cell-cell and cell-matrix interactions by binding respectively to vascular cell adhesion molecule-1 (VCAM-1), non-RGD sequences (Arg–Gly–Asp) of fibronectin (FN), or C1q-like domain of elastin microfibril interfacer-1 (EMILIN-1).[13-17] These features are reflected in the independent prognostic impact of CD49d expression in CLL.[18-20] Moreover, a role of CD49d in proliferative centres of tissue sites can be inferred by studies investigating the expression of CD49d in the bona fide highly proliferative compartment of peripheral blood CLL cells.[21] In particular, CD49d was expressed at a higher level in highly proliferative peripheral blood CLL cells, as defined by the CD5 high/CXCR4 low phenotype, than in cells of the resting compartment.[21]

In the present review, we focused on functional and physical interactions of CD49d with other microenvironmental receptors, including CD38 and BCR, and other specific CD49d-dependent interactions in lymph node and bone marrow microenvironments responsible for growth and survival-supporting signals, eventually influencing CLL prognosis and therapeutic options.

CLINICAL IMPACT OF CD49d EXPRESSION

Recently, in the context of a multicentre worldwide initiative analysing a CLL series of about 3,000 cases,[22] the expression of CD49d - the α chain of the $\alpha_4\beta_1$ integrin heterodimer - emerged as a first level biological prognosticator in CLL, predicting shorter overall survival and progression free survival, along with IGHV mutational status and deletion 17p.[18,23-27] On the contrary, CD38 and ZAP70, as well as the cytogenetic abnormalities deletion 11q and trisomy 12, evidenced a generally lower prognostic impact.

In the same study, when a hierarchical model was restricted to the flow cytometric prognostic markers CD49d, CD38, and ZAP70, CD49d was located at the top of the branching in the entire cohort of patients, as well as in early stages and young patients, thus resulting in the best flow-cytometry-based marker to stratify the prognosis of CLL patients.[22] Given this evidence, testing CD49d expression in routine clinical practice emerged as similarly useful in the baseline prognostic assessment of newly diagnosed CLL, as well as in refining the prognostic evaluation in patients already stratified by CD38 and/or ZAP70 expression. These clinical aspects could be considered as direct and specific consequences of physical and chemical interactions of the CD49d molecule.

CD49d INVOLVEMENT IN THE MICROENVIRONMENTAL CROSS-TALK AND SURVIVAL SIGNALLING

CLL is chraracterised by several functional interactions involving CD49d and specific chemokine-cytokine receptor/ligand pairs. In particular, cell adhesion of CLL cells via the CD49d/VCAM-1 pair, and the subsequent response of adherent CLL cells to the chemokines, CCL21 and CCL19, produced by high endothelial venules (HEV), or by the surrounding lymph node stroma through their receptor CCR7, is involved in transendothelial migration (TEM) of CLL cells across HEV into lymph nodes.[28] In addition, the combined stimulation of CLL cells by vascular endothelial cell growth factor (VEGF), and by CD49d engagement, was shown to be critical for TEM induced by CCL21 and CXCL12 in CLL cells coexpressing CD49d along with the VEGF receptors VEGFR1 and VEGFR2.[29]

Adhesion of CLL cells via CD49d also upregulates matrix metalloproteinase (MMP)-9 production, the MMP-9 proteolytic activity may be enhanced by its localisation at the CLL cell surface.[30] In particular, CLL cells bind soluble and immobilised pro-MMP-9 and active MMP-9 through a cell surface docking complex for MMP-9, composed by CD49d and a splice variant of CD44, conferring a metastatic phenotype that locally causes the growing of tumour cells, and whose expression is associated to tumour progression.[31] MMP-9 is also a functional ligand for the CD44v/CD49d docking receptor, able to provide survival signals independently of its proteolytic activity.[31,32] Interestingly, the pro-survival effect of MMP-9 derives from activation of the Lyn kinase, thus following a distinct and BCR-independent mechanism.[32] Moreover, the LYN/STAT3/MCL-1 pathway, which is elicited by MMP-9 ligation to the CD44v/CD49d docking receptor, is not shared by the CD49d-VCAM-1 axis, suggesting that CD49d may trigger distinct intracellular events depending on the ligand.[32]

CXCR4, the receptor for the CXCL12 chemokine, is also associated with CD49d on CLL cell membrane, suggesting that CD49d and CXCR4 may be functionally linked in CLL,[33] as demonstrated in multiple myeloma or in bone marrow hematopoietic progenitors, where CXCR4, triggering by CXCL12, is able to upregulate CD49d-mediated adhesion to VCAM-1 and FN.[34,35] Of note, as for CD49d, CXCR4 engagement was also shown to upregulate MMP-9 production by CLL cells.[30]

In CLL, ligation of CD49d by FN was demonstrated to prevent *in vitro* onset of apoptosis, likely due to an increase in the BCL-2/BCL-2-associated X protein (BAX) ratio,[36] and to protect CLL cells from fludarabine-induced apoptosis, this effect correlated with an increased expression of BCL_{XL}.[13,37] CD49d triggering is also able to induce spleen tyrosine kinase (SYK) phosphorylation and SYK-dependent protein kinase B (AKT) phosphorylation, through mechanisms distinct from the BCR signalling.[38] The SYK-dependent AKT/myeloid cell leukaemia sequence 1 (MCL-1) pathway is known to contribute to CLL cell survival.[39-42]

Co-culture of CLL cells with endothelial cells determines a significant increase of CD49d expression and enhances CLL cell viability, these effects being mediated by activation of the NF-κB transcription factor RelA.[43] The genes induced by NF-κB to promote survival include the cellular inhibitor of apoptosis FLIP, and the BCL-2 homologous A1 and BCL_{XL}.[44] Alterations in NF-κB signalling cascades have been considered responsible for the differences in the sensitivity to microenvironment stimuli between high and low-risk groups, such as CLL expressing unmutated IGHV and mutated IGHV, or CD49d positive ($CD49d^+$) and CD49d negative ($CD49d^-$) CLL.[45,46]

FUNCTIONAL INTERACTIONS OF CD49d WITH BCR

The binding of CLL cells on stromal cells of microenvironmental niches, mainly occurring through CD49d, reflects the activity of normal B cells where CD49d-driven interactions play a key role in controlling the development of B lymphocytes,[47,48] chemokine-induced transendothelial migration (TEM) of mature B cells during their recirculation and homing,[49,50] and antigen-specific B cell differentiation within germinal centres of secondary lymphoid organs.[51] In particular, during the latter process, B cells that express BCR with high affinity for the antigen are rescued from apoptosis by interacting with follicular DCs through the $\alpha_4\beta_1$/VCAM-1 axis.[52,53] This inside-outside activation of the $\alpha_4\beta_1$ integrin is BCR-controlled through the consecutive activation of LYN, SYK, PI3K, BTK, PLCγ2, IP3R, and PKC. In particular, upon BCR stimulation, $\alpha_4\beta_1$ can be released from a cytoskeletal constraint by Ca^{++}-mediated BCR-dependent calpain activation and mobilised to lipid rafts, this process leading to the formation of $\alpha_4\beta_1$ clusters that, in turn, may become tethered to the actin cytoskeleton, eventually resulting in enhanced $\alpha_4\beta_1$ avidity and adhesion.[54-56] In this model, B cells expressing BCR with high affinity for the presented antigen are preserved in the germinal centre by integrin-mediated signals while, on the contrary, B cells expressing BCR with low affinity for the presented antigen, failing to have sufficient integrin mediated signals, are more prone to apoptosis.[57]

The described BCR-dependent $\alpha_4\beta_1$ functional interaction can be preserved in CLL, where the increased lymph node size is mainly/exclusively dependent from the accumulation of CLL cells due to integrin mediated adhesion to accessory cells and/or extracellular matrix proteins.[57] Inhibitors of kinases, downstream of the BCR such as the Bruton's tyrosine kinase (BTK) inhibitor ibrutinib,[58] the SYK inhibitor fosfamatinib,[59] and the PI3Kδ

inhibitor idelalisib,[60] are promising alternative target therapies for CLL patients that have been very recently employed in clinical trials. These new agents have a clinical activity that appear similar, with a rapid resolution of lymphadenopathy and/or organomegaly, and a redistribution of CLL cells from tissue into the blood, with a subsequent rising of lymphocytosis during the first few weeks of therapy that often slowly resolves. In this context, it has been reported that: 1) the BTK inhibitor ibrutinib strongly inhibits this CD49d-mediated adhesion of CLL cells to VCAM-1 and FN substrates *in vitro*;[61] 2) the PI3Kδ inhibitor idelalisib decreases CLL adhesion to stromal cells by interfering with CD49d/VCAM-1 binding.[62] Thus, these mechanisms of action could be the cause for lymph node shrinkage with the redistribution of CLL cells into the blood observed *in vivo* upon treatment of CLL patients with BTK inhibitors,[61,62] and may also provide the rationale for the use of inhibitors of kinases in combination therapies aimed at targeting CLL cells outside the microenvironmental niches, where they may be more prone to respond to immuno-chemotherapy.

CD49d INTERACTIONS WITH CD38

A functional link between CD49d and CD38 involves CCL3 and CCL4 chemokines that have been found to be overexpressed in CD49d$^+$CD38$^+$ CLL cells, and upregulated upon CD38 triggering.[63] CLL-derived CCL3 and CCL4 have been associated with the recruitment of cells from the monocyte macrophage,[63,64] or T cell lineages,[65] in the context of CLL-involved microenvironmental sites.[63,64,66,67] Moreover, a strong correlation among the CD49d$^+$CD38$^+$ phenotype, infiltration of CD68$^+$ macrophages, and presence of a stromal/endothelial component highly expressing VCAM-1 in the context of lymphoid aggregates in bone marrow biopsies of CD49d$^+$CD38$^+$ CLL, has been demonstrated.[63] In particular, VCAM-1 upregulation has been found due to an overproduction by the infiltrating CD68$^+$ macrophage component of TNF-α, allegedly together with other cytokines.[63] This circuitry may contribute to explain the aggressive clinical course of CLL coexpressing CD49d and CD38, given the pro-survival effects of VCAM-1/CD49d interactions for CD49d-expressing CLL cells.[63]

In CLL, CD49d and CD38 are part of a cell surface macromolecular complex which also includes CD44 and MMP-9, as well as CXCR4,[68] thus characterising a signalling platform in CLL cells of poor prognosis cases. The association between CD38 and the CD49d/CD29 integrin heterodimer, both inside and outside the cell membrane lipid rafts, allows the CD49d/CD29/CD38 complexes to freely shuttle in and out of the specialised cholesterol enriched membrane microdomains, where signalling transduction is organised.[69,70] Moreover, the CD49d/CD29/CD38 complexes are not influenced by the integrity of the membrane structure since the association is unaffected by cholesterol depletion, being joined together by other cellular structures, including cytoskeletal proteins, known to associate with integrins either directly or indirectly.[71]

CD49d-mediated activities are enhanced by the co-expression of CD38; in fact, CD49d$^+$CD38+ cells have higher propensity to adhere and to spread when seeded onto the CD49d-specific substrates VCAM-1 and FN compared to CD49d$^+$CD38$^-$ cells. In this context, CD49d/VCAM-1 interactions exert a more marked anti-apoptotic effect in CD49d$^+$CD38$^+$ as compared to CD49d$^+$CD38$^-$ cells. Moreover, adherent CD49d$^+$CD38$^+$ CLL cells display a distinctive morphology, characterised by a more complex pattern of filopodia-like protusions compared with cells with the CD49d$^+$CD38$^-$ phenotype.[72] CD38 was also demonstrated to be effective in the recruitment of Vav-1, a molecule involved in the integrin pathway, that operates as guanine exchange factor for Rac and Cdc42, two Rho GTPases involved in lamellipodia/filopodia generation in various cell models,[73-75] and that becomes phosphorylated on tyrosine-174 upon integrin engagement. Of note, CD49d$^+$CD38$^+$ CLL cells are characterised by higher levels of phospho-Vav-1 upon adhesion onto CD49d-specific substrates than CD49d$^+$CD38$^-$ CLL cells, resulting in a more robust integrin signalling pathway characterising CD49d$^+$CD38$^+$ CLL.

The physical association between CD49d and CD38 is also responsible for a more marked anti-apoptotic effect exerted upon CD49d/VCAM-1 interactions in CD49d$^+$CD38$^+$ CLL cells than in CD49d$^+$CD38$^+$ CLL cells. This characteristic can depend on a more efficient adhesion of CD49d$^+$CD38$^+$ CLL cells, and consequently a more pronounced activation of the anti-apoptotic machinery,[13,63] also, thanks to the contribution of specific signalling proteins, such as Vav-1,[76] already recruited to the adhesion site.

ASSOCIATION OF CD49d WITH TRISOMY 12

In a recent study by our group,[77] CD49d expression was investigated by flow cytometry in the neoplastic component of 1,200 CLL patients. In this series, using the cut-off of 30% of positive cells, about 40% of cases were classified as CD49d$^+$ cases. Analysis within the major cytogenetic groups showed that a significantly higher percentage of CD49d$^+$ cases (about 90% of cases) is associated with the presence of trisomy 12 cases. Moreover, among CD49d$^+$ cases, trisomy 12 CLL cases are characterised by the higher mean fluorescence intensity levels when compared with cases belonging to the other cytogenetic categories. Additionally, in the context of flow cytometry sorted CD49d$^+$ and CD49d$^-$ subpopulations in CLL cases with bimodal CD49d expression, trisomy 12 abnormality could be detected only in the CD49d$^+$ fraction and it was absent in CD49d$^-$ cells.

In the same study, DNA methylation was analysed within a 5'-UTR CpG island (77 CpGs) of the CD49d gene (ITGA4).[77] In this context, it was found that: 1) CD49d$^+$/trisomy 12 CLL virtually completely lacked methylated CpG, while a significant methylation of CpG was detected in CD49d$^-$ cases; 2) a significant inverse correlation was found between the percentage of methylated CpGs and CD49d expression at both mRNA and protein levels; 3) when highly purified CLL cells from CD49d$^-$ cases were exposed to the hypomethylating agent 5-aza-2'-deoxycytidine (DAC) in the presence of CpG-ODN/interleukin-2 as a proliferative stimulus, the proliferative fraction of DAC treated CLL cells, significantly upregulated CD49d protein levels; 4) consistently, analysis of ITGA4 methylation in these DAC treated proliferating cells revealed lower levels of DNA methylation in ITGA4 5'-UTR CpG-island compared with proliferating CLL cells of untreated cultures.

Overall, these data highlight a direct role of DNA methylation in regulating CD49d expression in CLL. Moreover, the overexpression of CD49d may contribute to explain: 1) the molecular basis of the peculiar biological behavior of trisomy 12 CLL and may predict for the development of additional cytogentic lesions;[78] and 2) the specific tropism toward lymph nodes of trisomy 12 CLL cells and the peculiar clinical features of this CLL subset, in which massive lymph node enlargement is often observed and the final transformation in Richter's syndrome is more frequent than in other cytogenetic categories.[79]

CONCLUSION

In the present review, we have summarised the principal microenvironmental interactions involving CD49d in CLL. In fact, CD49d can be represented as a major factor of a complex interplay with other surface receptors, all expressed by CLL cells, which are able either to potentiate CD49d activities (e.g. CD38, CXCR4, VEGFR1/2, BCR) or are potentiated by interactions with CD49d itself (e.g. CD44, CXCR7). As a consequence of CD49d engagement, pro-survival signals and signals protecting CLL cells from drug-induced damages are delivered (Figure 1).

An interesting observation is the strong correlation between CD49d expression and trisomy 12 since it might anticipate a putative general feature of CLL cells, i.e. the non-random correlation between genetic lesions and microenvironmental receptors. In this context, recent studies by us and others[3,4,80-83] reported the non-random association of specific BCR features, i.e. the expression of the so-called stereotyped BCR, with the novel somatic mutations with prognostic relevance of genes such as NOTCH1 and SF3B1.

The characteristic clinical activity of kinase inhibitors targeting BCR downstream genes consisting in CLL cell redistribution from tissues into the blood emphasise a relevant role for CLL microenvironment not only in CLL pathogenesis but also in the development of new targeted treatment approaches. In particular, the employment of such inhibitors, being non-genotoxic compounds, could also be useful in the context of asymptomatic patients, in which a potential selection of genomic alterations due to DNA-damaging chemotherapy must be avoided, and in which the usual approach is a watch and wait strategy. In this context, the relevance of CD49d expression should be tested in clinical trials similar to the trial planned by the German CLL study group[5] in which ibrutinib is employed as a first-line treatment in patients with early stages of disease (e.g. Binet Stage A). In conclusion, the complex network of CD49d-mastered microenvironmental interactions and/or correlations, as detailed in the present review, may have a relevant role that remains to be established and will be addressed by future studies.

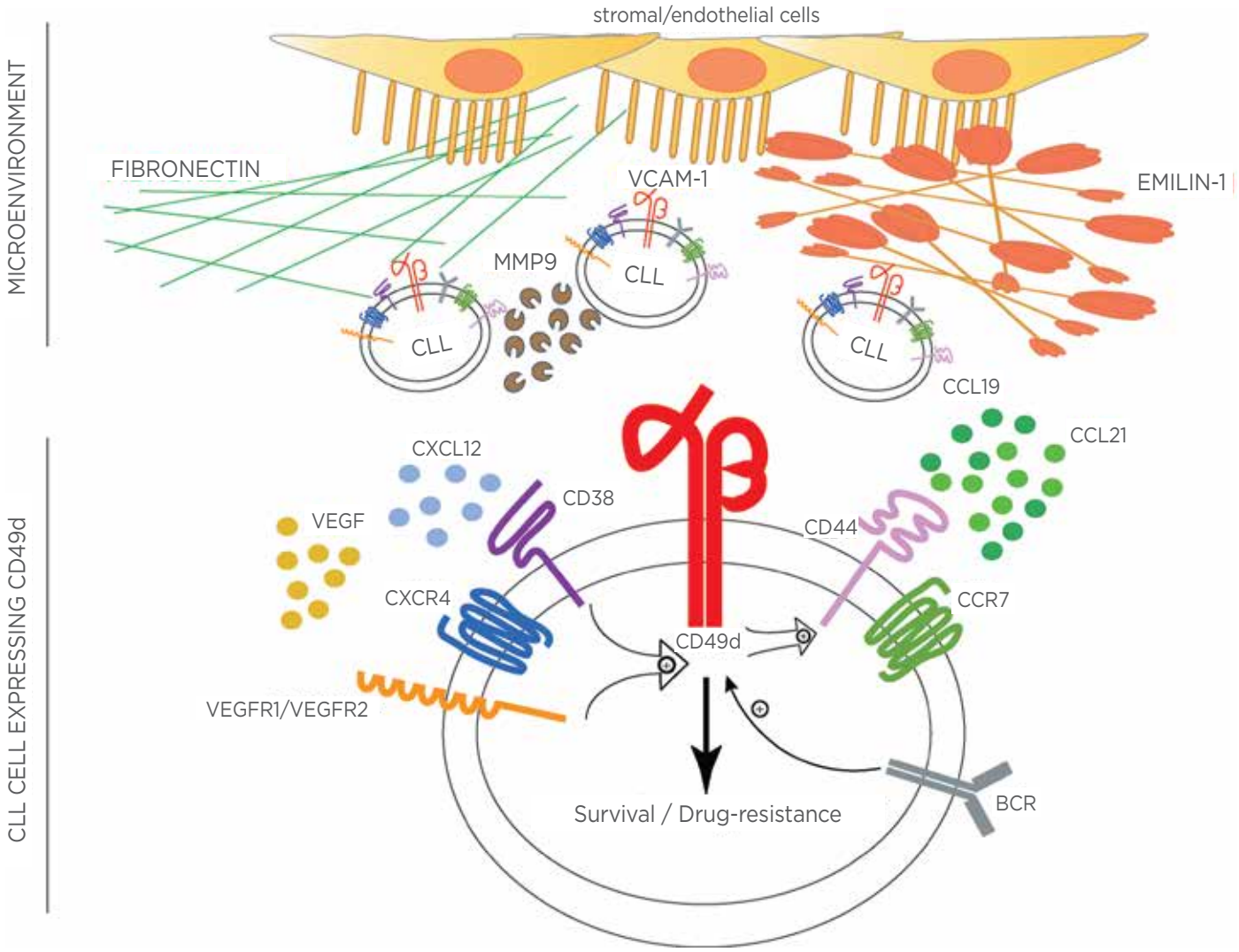

Figure 1: CD49d interactions in CLL microenvironment.
CLL: chronic lymphocytic leukaemia; VCAM-1: vascular cell adhesion molecule-1; EMILIN-1: elastin microfibril interfacer-1; MMP: matrix metalloproteinase; VEGF(R): vascular endothelial cell growth factor (receptor); BCR: B cell receptor.

Acknowledgements

Supported, in part, by: the Associazione Italiana Ricerca Cancro (AIRC), Investigator Grant IG-13227, MFAG-10327; Progetto Ricerca Finalizzata I.R.C.C.S. n. RF-2009-1469205, n. RF-2010-2307262, Progetto Giovani Ricercatori n. GR-2009-1475467, Ministero della Salute, Rome, Italy; Fondazione Internazionale di Ricerca in Medicina Sperimentale (FIRMS) Turin, Italy; Associazione Italiana contro le Leucemie, linfomi e mielomi (AIL), Venezia Section, Pramaggiore Group, Italy; Ricerca Scientifica Applicata, Regione Friuli Venezia Giulia ("Linfonet" Project), Trieste, Italy; "5x1000 Intramural Program", Centro di Riferimento Oncologico, Aviano, Italy.

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