CD38 in Chronic Lymphocytic Leukemia: From Bench to Bedside?

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Abstract: Human CD38 is a cell surface molecule endowed with multiple functions. As an enzyme, it catalyzes the production of Ca^{2+} active metabolites, predominantly cADPR and ADPR. As a receptor, it regulates the activation of an intracellular signaling pathway, generally linked to lymphocyte activation and proliferation in physiological conditions. The finding that CD38 behaves as an independent negative prognostic factor in CLL patients was the starting point for investigations into the functional role of the molecule in the neoplastic context. Data accumulating in over a decade concur to define a model where CD38 is a central element of a large supramolecular complex that includes surface signaling receptors, chemokine receptors, adhesion molecules and matrix metalloproteases. Expression of CD38 within this supramolecular complex makes signal transduction as well as chemotaxis and homing more efficient, suggesting that the molecule is an integrator of proliferative and migratory signals. These data indicate that CD38 is not only a reliable disease marker but also a functional molecule in the CLL context. The next decade will likely tell whether it can also be a useful therapeutic target.

Keywords: Chronic lymphocytic leukemia, CD38, CD31, proliferation, chemotaxis, homing.

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is the result of the expansion of a population of mature B lymphocytes characterized by the expression of CD5 [1]. Diagnosis is relatively simple, but predicting the clinical outcome may be problematic. Approximately one-third of patients are affected by an indolent form of the disease that does not require treatment or impact on the survival. On the other hand, another significant proportion of patients are characterized by an aggressive form of leukemia that needs to be repeatedly treated, with severe consequences on the quality of life and survival. The remaining third shows intermediate disease features [2]. The necessity of distinguishing between the two extremes (indolent *vs* aggressive) was the starting point for the quest for specific prognostic parameters that can be used at diagnosis [3]. Several clinical and molecular markers have been identified that characterize the aggressive variant of the disease. The surface molecule CD38 is one of them: its expression has proven a reliable and independent negative prognostic marker, consistently characterizing patients with unfavorable prognosis [4]. The correlation between CD38 expression and the absence of mutations in the IgVh genes is not as strong as originally believed [5]. However, their concordant presence is a better indicator of the aggressive form of disease than either marker used alone [6]. Moreover, CD38 expression correlates with other negative prognostic markers for the disease, such as Zap-70 [7], cytogenetic abnormalities [8], soluble β2m [9], soluble CD23 [10] and p53 function [11].

CLL is now considered as a disease characterized by a dynamic balance between proliferating cells located in the lymphoid organs and circulating cells resisting programmed cell death [12]. The balance is tuned through interactions between the tumor and the host, implying that the set of surface molecules expressed by the leukemic cell may change the nature of these interactions, promoting proliferation over resistance to apoptosis [13]. In line with the hypothesis that CD38 is one of the players in the communication network between tumor and host, its expression by CLL cells is higher in peripheral lymphoid organs and in bone marrow than in circulating CLL cells of the same patient [14]. Moreover, experimental data indicate that CD38 delivers growth and survival signals to CLL cells and influences migration in conjunction with chemokines and their receptors [15-17]. The translational implication is that this network of signals may become a therapeutic target, with specific monoclonal antibodies (mAbs) or small molecules used in combination with conventional chemotherapy [18].

BIOLOGY OF HUMAN CD38

Human CD38 is a type II surface glycoprotein characterized by a large extracellular domain, a single transmembrane pass and a short cytoplasmic tail [19]. Within the human immune system, it is expressed by

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immature hematopoietic cells, down-regulated by mature lymphocytes and re-expressed at high levels by activated T and B, dendritic and natural killer (NK) cells [20]. CD38 is a bi-functional molecule endowed with enzymatic and receptor functions. As an enzyme, CD38 belongs to the ADP ribosyl cyclase (ADPRC) family, seemingly ubiquitous in eukaryotic cells [21]. By generating the second messenger cyclic ADP ribose (cADPR) from nicotinamide adenine dinucleotide (NAD), ADPRCs regulate a wide range of physiological processes. The enzymatic site of CD38 is contained in the extracellular domain and is multifunctional. It can synthesize cADPR from ADPR, although the major enzymatic product of CD38 is ADPR, which can be generated from NAD^+ or cADPR through hydrolysis. CD38 can also use $NADP^+$ as a substrate to generate nicotinic acid adenine dinucleotide phosphate (NAADP). All these products are involved in signal transduction through the modulation of cytoplasmic Ca^{2+} concentrations [22].

Besides being an enzyme, CD38 has well characterized receptor properties. Following the interaction with CD31, a non-substrate ligand expressed by a variety of immune cells and by vascular endothelial cells [23], CD38 induces activation, proliferation and cytokine release in selected lymphocyte subsets. The use of agonistic (*i.e.*, eliciting activatory signals) mAbs against the molecule demonstrated that CD38 engagement is followed by the activation of a substrate-independent signaling cascade [20]. The essential steps of the pathway involve increase in intracellular Ca^{2+} and tyrosine phosphorylation of a sequential signal transducers. Long-term effects depend on active protein synthesis. Transmission of signals through CD38 is tightly regulated at distinct levels. The first involves the structural organization of CD38, divided into mono- and multi-meric forms, with distinct enzymatic and receptor functions. The second is based on the dynamic localization of the molecule in lipid microdomains within the plasma membrane [24, 25]. A significant fraction of the membrane pool of CD38 is constitutively localized in cholesterol-rich regions, while the remaining CD38 molecules join the raft pool upon engagement with agonistic mAbs [26]. Disruption of the rafts prevents signal transduction through CD38. The third level of control is determined by lateral associations on the same cell with other proteins or protein complexes, which include antigen receptors, adhesion molecules, chemokine receptors and tetraspanins. Lastly, recent data indicates the presence of distinct pools of CD38 molecules, not only on the cell surface, but also in internal compartments (endoplasmic reticulum, endosomes, and nucleus) and in secreted exosomes [27]. The specific contribution of each of these CD38 stores to signal transduction remains unclear, even though it has been shown that upon antigen recognition, intracellular CD38 molecules in T lymphocytes very rapidly reach the cell surface and become available at the immunological synapse interphase [28].

HIGH CD38 EXPRESSION CHARACTERIZES A DISEASE SUBSET WITH AGGRESSIVE BEHAVIOR

The first evidence linking CD38 to functionally and clinically possible distinct CLL subgroups was suggested by the finding that the functional responses following IgM engagement were dependent on CD38 expression [29, 30]. These observations were confirmed when considering the $CD38^+$ fraction of a single clone, characterized by increased response to antigen signaling through the B cell receptor (BCR) as compared to the $CD38^-$ one. This was concluded after observing that the $CD38^+$ subpopulation expressed more ZAP-70, had more robust protein tyrosine phosphorylation and a greater propensity to apoptosis upon BCR cross-linking than the corresponding counterpart [31]. These findings suggest a function for CD38 in the modulation of BCR signals and in promoting antigen-driven CLL cell expansion. Furthermore, CD38 labels the proliferating fraction of the clone, as seen from the selective enrichment of Ki-67-expressing cells [32]. Gene expression profiling and protein analysis of highly purified cell-sorted $CD38^+$ and $CD38^-$ components of a single clone indicated that $CD38^+$ CLL cells possessed a distinct gene profile, with increased expression of genes involved in BCR signaling, angiogenesis and lymphomagenesis [33].

In vivo labeling experiments using deueterated water (^2H_2O) drank by CLL patients were used to measured disease kinetics. Results indicated that a measurable proportion of the clone undergoes daily renovation. In every patient a larger enrichment of newly produced $CD38^+$ than $CD38^–$ cells was found, substantiating that the two subclones have a different proliferative kinetics [34].

CD38 AND CLL: WHERE CELL PROLIFERATION AND MIGRATION CONVERGE

Several pieces of evidence suggest that CD38 actively affects expansion and proliferation of the neoplastic clone. First, CD38 engagement by means of agonistic mAbs is followed by proliferation and blast transformation of a subset of CLL cells, proving that the molecule performs as an active signaling receptor [35]. The simultaneous presence of IL-2, which strongly up-regulates CD38 expression, significantly enhances the *in vitro* signaling properties of the molecule. Second, the CD31 ligand similarly activates the signaling cascade [36]. $CD38^+$ CLL cells juxtaposed to murine fibroblasts transfected with CD31 in an *in vitro* model mimicking microenvironmental interactions exhibit increased growth, survival and propensity to migrate in response to CXCL12 chemokine. Furthermore, CD38/CD31 interactions up-regulate CD100, a survival receptor of the semaphorin family involved in sustaining CLL growth. These results are indirectly confirmed using nurse-like cells derived from CLL patients, which express high levels of functional CD31 and plexin-B1, the high-affinity ligand for CD100 [37].

A crucial step in CLL progression is the recirculation of neoplastic cells from blood to lymphoid niches, where proliferative and survival signals are provided [38]. In the CLL model, chemokines significantly contribute to the delivery of growth signals to CLL cells expressing functional receptors. The key question is whether CD38 can be a point of convergence between proliferative and migratory signals, contributing to the homing of CLL cells. Independent data from murine models indicate that CD38 regulates homing of immune cells. Indeed, *CD38*-deficient mice are characterized by impaired chemotactic responses to distinct chemokines, suggesting a role for the molecule in the sensing of the

chemokine and in the priming of the cell for movement [39, 40]. The analysis of chemotactic responses to CXCL12, the main chemokine involved in CLL cell recirculation to and from lymph nodes, demonstrate that $CD38^+$ CLL cells are more responsive than their negative counterparts [41]. These results were confirmed in CLL patients with a bimodal expression of CD38. The subclone lacking the molecule was significantly less responsive to the chemokine than the intact clone. Moreover, patients with the highest sensitivity to CXCL12 co-expressed CD38 and ZAP-70. This kinase is directly phosphorylated upon CD38 cross-linking and represents a limiting factor in the CD38 pathway [41]. These evidence may help explain why CLL patients who simultaneously express CD38 and ZAP-70 perform consistently worse than those expressing each marker alone [42]. As a further consideration, ZAP-70 appears to be a signaling element shared by CD38 and CXCR4, suggesting functional cooperation between the two molecules. A synergism between these two molecules was shown by modulating chemotaxis with agonistic or blocking anti-CD38 mAbs. The engagement of CD38 by means of agonistic mAbs results in enhanced chemotaxis, while blocking reagents significantly impaired homing of CLL cells, both *in vitro* and *in vivo* conditions [43]. These results may be explained by the physical proximity of CD38 and CXCR4 on the cell membrane. Indeed, confocal microscopy revealed that CD38 and CXCR4 co-localize, at least in part, in the same membrane patches. Different results are provoked upon chemokine administration: CXCR4 is partially internalized, whereas CD38 is apparently unaffected, suggesting that the molecules follow different pathways [43].

The homing process involves the functional cooperation of several molecules, such as chemokines and their receptors, integrins and proteases able to digest the extracellular matrix (Fig. **1**). Operating in association with chemokine receptors, integrins are cell surface adhesion proteins that connect the plasma membrane to the actin cytoskeleton. Experimental data suggest that CD31/CD38 signals are part of a molecular circuit involving the CCL3 and CCL4 chemokines and the integrin CD49d (α_4) [44]. The α_4 integrin is a further negative prognostic marker for CLL patients and through the binding to vascular cell adhesion molecule-1 (VCAM-1) regulates CLL cell extravasation [45]. Experimental data shows that CD31/CD38 interactions result in the rapid release of a panel of chemokines by CLL cells. The final effect is the expression of VCAM-1 by stromal and endothelial cells, with the activation of pro-survival signals in CLL cells mediated by CD49d/VCAM-1 axis [44]. As demonstrated with CXCR4, the functional link between CD38 and CD49d depends on their physical association on the CLL cell membrane, as inferred by co-localization and co-immunoprecipitation experiments, which implies the existence of a large supra-molecular complex. The complex is dynamic and the association appears to be strengthened when CLL cells are left to adhere on recombinant human VCAM-1 [16].

The *in vivo* molecular network regulating CLL cell homing includes other players, such as matrix metalloproteases (MMPs). MMPs, in physiological conditions, have a central role in the extracellular matrix turnover and are well-known modulators of the tumor microenvironment, contributing to tissue invasion and metastasis. In the CLL model, MMP-9 is the predominant gelatinase expressed, and its intracellular levels correlate with advanced stage of the disease and poor patient survival

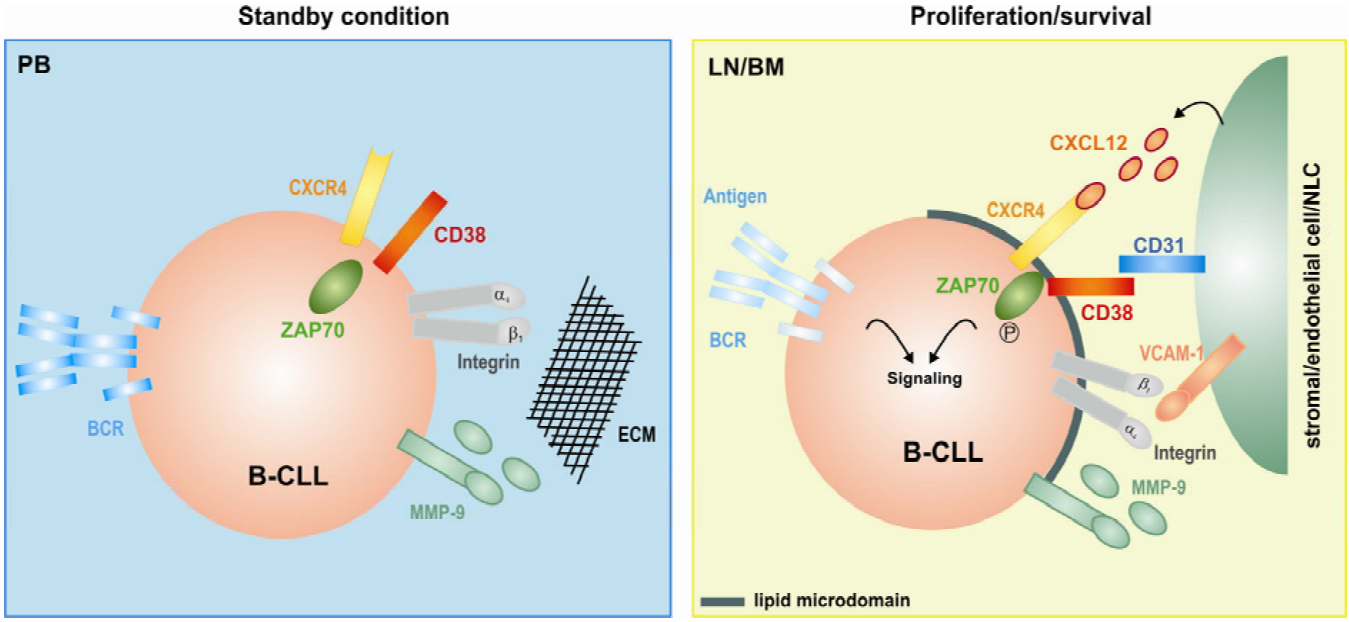

Fig. (1). Cartoon depicting microenvironmental influences and interactions of CLL cells in the circulation (PB, left panel) and in lymphoid organs (LN/BM, right panel). According to this model, CD38 is a central element in a large supra-molecular complex that includes chemokine receptors, adhesion molecules and matrix metalloproteases, among the others. Lymphoid tissues provide accessibility to the ligands of the different molecules, switching on the circuit. The final outcome is an increased propensity of the leukemic cell to respond to antigen-mediated signals.

[46]. Recent data highlighted the involvement of MMP-9 in CLL cell migration and survival and suggested that the proteolytic activity may be enhanced by its localization on the CLL cell surface. CD49d/CD29 integrins and the "v" variant of CD44 (CD44v) were identified as docking molecules for MMP-9 [47]. CD44 isoforms are a family of transmembrane receptors for hyaluronic acid (HA), a major component of the extracellular matrix, and are involved in selected adhesion functions and bidirectional (outside to inside and vice versa) signals. The involvement of CD44v as a potential regulator of CLL chemotaxis through modulation of MMP-9 localization is intriguing, particularly in light of a previous observation suggesting that CD38 might also be involved in the binding of glycosaminoglycans, sharing them with CD44 [48].

Taken together, these results suggest the presence of a macro-molecular complex on CLL membrane that includes CD38, chemokine receptors, integrins and likely matrix metalloproteases. The functional cooperation of these molecules, mediated by CD38, results in a higher ability of neoplastic cells to migrate from blood to lymphoid organs.

From the above data, it is clear that CD38 is directly part of the pathogenetic network underlying CLL development and progression. These characteristics along with its membrane localization render CD38 a potentially attractive therapeutic target, also exploiting the availability of specific antibodies or soluble ligands, good candidates to specifically bind the molecule [49]. The next decade will likely tell whether these promises will be fulfilled.

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