



# The role of CD44 in the pathophysiology of chronic lymphocytic leukemia

Julia Christine Gutjahr<sup>1,2</sup>, Richard Greil<sup>1,2</sup> and Tanja Nicole Hartmann<sup>1,2\*</sup>

<sup>1</sup> Laboratory for Immunological and Molecular Cancer Research, 3rd Medical Department with Hematology, Medical Oncology, Hemostaseology, Infectiology and Rheumatology, Oncologic Centre, Paracelsus Medical University, Salzburg, Austria

<sup>2</sup> Salzburg Cancer Research Institute, Salzburg, Austria

## Edited by:

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## \*Correspondence:

Tanja Nicole Hartmann, Laboratory for Immunological and Molecular Cancer Research, 3rd Medical Department with Hematology, Medical Oncology, Hemostaseology, Infectiology and Rheumatology, Oncologic Centre, Paracelsus Medical University, Muellner Hauptstrasse 48, Salzburg 5020, Austria  
e-mail: t.hartmann@salk.at

CD44 interactions with hyaluronan (HA) play a key role in various malignancies, supporting tumor cell migration, adhesion, and survival. In contrast to solid tumors, the expression of CD44 standard and variant forms and their functional interplay with HA is less understood in hematological malignancies. Chronic lymphocytic leukemia (CLL) is a highly abundant B-cell malignancy with a well coordinated balance between cell cycle-arrest and proliferation of tumor subpopulations. The long-term survival and proliferation of CLL cells requires their dynamic interactions with stromal and immune cells in lymphoid organs. Interactions of HA with CD44 and HA-mediated motility receptor (RHAMM) contribute to CLL cell localization, and hence CLL pathophysiology, by shaping homing, interstitial migration, and adhesion of the tumor cells. CD44 can complex with key prognostic factors of CLL, particularly CD38 and CD49d, bridging the gap between prognosis and cellular function. Here, we review the current evidence for the individual and associated contributions of CD44 to CLL pathophysiology, the dynamic functional regulation of CD44 upon CLL cell activation, and possible therapeutic strategies targeting CD44 in CLL.

**Keywords:** CD44, chronic lymphocytic leukemia, microenvironment, homing, hyaluronan

## INTRODUCTION

The tumor microenvironment, shaped by interactions between malignant and non-malignant cells, is influential for tumor formation and progression of various cancers. Chronic lymphocytic leukemia (CLL) is a disease of mature B lymphocytes and is manifested by progressive accumulation of these malignant cells in blood, bone marrow (BM), and lymphoid tissues (1). Characteristically, CLL follows extremely variable clinical courses with survival times ranging from months to decades, making it necessary to classify the patients according to prognostic risk (2). Besides genomic aberrations such as 17p deletion, 13q deletion, trisomy 12, and 11q deletion, a most important and long-established prognostic marker is the mutational status of the B-cell receptor (BCR) immunoglobulin variable heavy chain (IgVH) genes (2–4). Patients with CLL cells that express IgVH genes without significant levels of mutation (<2% difference from germline gene counterpart, “unmutated”) follow a more aggressive clinical course with shorter times to first treatment and overall survival than patients harboring IgVH gene mutations (≥2% difference from germline, “mutated”) (5). Other common prognostic parameters are the extent of expression of CD38 and zeta-chain-associated protein kinase 70 (ZAP-70), earlier suggested as surrogate markers for the IgVH mutation status (6). However, both have independent prognostic power, too.

CD49d, despite being the newest among the prognostic markers, is the strongest flow cytometry-based predictor of overall survival and treatment-free survival in CLL (7). Following the first reports on the poor outcome of patients with an expression

of CD49d on ≥30% of the tumor cells (8,9), its high prognostic relevance has been unequivocally confirmed by several groups (10–15). Expression of CD38 and CD49d is associated in about 80% of samples (12,16) and the molecules can form macromolecular complexes with CD44 (17,18).

It is well established that the CLL pathophysiology relies on the lymphoid tumor microenvironment. Unusual for tumor cells, CLL cells circulating in the peripheral blood are cell cycle arrested. *Ex vivo*, CLL cells rapidly die from apoptosis if not co-cultured with immune or stromal cells, suggesting that the malignant cells are in constant need of supportive signals from the lymphoid microenvironment (19). It is therefore believed that at least a subpopulation of the peripheral blood CLL pool is able to recirculate into lymphoid organs in order to receive signals for proliferation and survival. Moreover, retention in these organs appears to favor onset and progression of CLL. Consequently, therapeutic targeting the microenvironmental interactions and lymphoid localization of the malignant cells emerges as a most successful strategy to permanently control CLL. This is impressively reflected by the clinical success of novel drugs such as ibrutinib and idelalisib that inhibit downstream signals of the BCR and retention molecules (20–23). Notably, the mode of action of ibrutinib and idelalisib is likely dual, they antagonize tumor cell proliferation in a NF-κB dependent manner (24,25) and disrupt CLL cell retention in lymphoid organs. Particularly, during the first period of treatment with these drugs, a redistribution of CLL cells from the lymphoid organs into the peripheral blood of patients can be observed (21,26,27), obviously depriving the tumor cells of supportive signals.

Despite this recent therapeutic progress, the detailed mechanisms that underlie the communication of CLL cells and accessory cells in the lymphoid microenvironment are still far from understood. Adhesion molecules and homing receptors orchestrate the localization and retention of CLL cells in lymphoid proliferation areas where CLL cells receive activation and protection signals. The glycoprotein CD44 can direct microenvironmental communication and intracellular signaling for growth and motility in many types of cancers (28). On hematopoietic cells, CD44 is universally expressed (28). The CD44 gene encodes different CD44 variant (CD44v) isoforms, which are generated by alternative splicing. The standard isoform of CD44 (CD44s) lacks the entire variable region. Hyaluronan (HA), the main ligand of CD44, is bound via a conserved BX<sub>7</sub>B binding motif (in which B represents Arg or Lys and X7 represents any seven non-acidic amino acids, but includes an additional Arg or Lys) present in the extracellular part of CD44 (28). The binding ability of the ubiquitously expressed molecules CD44 and HA needs to be strictly controlled. This can be achieved by posttranslational modifications such as glycosylations, CD44v expression, or CD44 clustering (28). In CLL, an external activation stimulus leads to increased CD44v expression and N-linked glycosylation, which induces CD44–HA binding (29). Concordantly, many studies have implicated CD44v rather than CD44s in tumor progression, dependent on the stage of progression and type of tumor (28).

In CLL, elevated CD44s and CD44v serum levels have been suggested as markers for disease progression and potential functional contributions to the pathophysiology have been discussed; however, the underlying biological mechanisms remain elusive. With some aspects controversially described, it has become necessary to further examine and more deeply understand the role of CD44 in this disease. Here, we discuss the prognostic role of CD44 and CD44v, its involvement in localization of CLL cells in lymphoid organs and tumor cell survival, and its suitability for therapeutic exploitation.

### HA RECEPTORS AND CLL PROGNOSIS

CD44 is described to form a complex with the prognostic markers CD49d and CD38, outlined in the introduction (18, 30). However, first reports on an individual prognostic role of CD44 in CLL were already published in the early 1990s (31), long before this complex was found. Despite this early discovery, the existing data are not completely consistent. In 1993, de Rossi and colleagues distinguished three groups of CLL patients, depending on either high, intermediate or low CD44 surface expression, defined in relation to the CD44 expression on T-cells. In this study, patients of the CD44-high group presented with an increased incidence of diffuse BM infiltration, which is a negative prognostic marker itself (31–33). Illogically, the follow up study of the same group identified these CD44-intermediate/high classified patients as good clinical outcomes (34). Subsequently and more consistent to the early findings, Eisterer and colleagues confirmed the prognostic value of CD44 by immunohistochemistry of BM specimen. CD44-high patients presented with advanced disease, a diffuse pattern of BM infiltration, and reduced survival within the observation period (35). Much later, Herishanu et al. (36) suggested that IgVH unmutated CLL cases express higher CD44 expression (36). We did not

find any differences in the intensity of CD44s expression in low and high risk patients, stratified according to IgVH mutation status, CD38, ZAP-70, or CD49d expression (29). This was confirmed by Fedorchenko et al. (37) when grouping patients according to IgVH mutational status or ZAP-70 expression.

The reason of these diverging observations remains unclear but one could hypothesize a differential activation status of the samples. We found that CD44 surface expression of CLL cells is induced upon their stimulation with activated T-cells or CD40 Ligand (CD40L) (29). In addition, several variant isoforms of CD44, known as markers for tumor progression in various malignancies (28), are transcribed and expressed at the surface upon activation (29).

In resting CLL cells, however, surface expression of CD44v is only detectable in the minority of CLL cases (38). These cases differ from the CD44v low expressing cases in regard to disease progression, lymphocyte doubling time, and therapy requirement (39). We found transcripts of CD44v3, v5, v6, v7, v8, v9, and v10 in unstimulated CLL cells, and a robust upregulation of CD44v3 and v6 upon CLL cell activation (29).

Soluble CD44, lacking the transmembrane region (40, 41) is found in serum due to shedding events (40, 42). High serum levels of CD44s, elevated in approximately half of CLL samples, are significantly associated with high tumor burden and the presence of other unfavorable prognostic markers such as high beta2-microglobulin levels (38, 43). The correlation is stable in time, treatment independent, and allows separation of two distinct patient groups with differential survival times (38). While de Rossi and colleagues did not observe any differences in CD44v in serum of CLL patients compared to healthy donors (38), a later study by Eisterer and colleagues identified elevated serum CD44v6 levels being associated with advanced disease defined by lymph node involvement and splenomegaly, and therapy requirement (44). This divergence was attributed to differential sensitivities of the statistical tests used. Nevertheless, independent analyses are required to solve these issues, particularly in case of CD44v6.

In CLL, little is known on the role of the second major HA-binding molecule RHAMM. One report describes a prominent expression of RHAMM and its splice variant RHAMM<sup>-exon 4</sup> in advanced CLL (45). As RHAMM expression was missing in peripheral blood mononuclear cells (PBMCs) from healthy individuals, it was suggested as a tumor-associated antigen (TAA) in CLL (45). A follow up study provided evidence of an additional prognostic role of RHAMM expression among CLL patients with mutated IgVH genes (46).

### MIGRATION AND LOCALIZATION

The CD44 molecule was originally defined as a lymphocyte homing receptor that can be bound by the Hermes class of antibodies (47–49). Homing hereby means the rapid process, in which circulating hematopoietic cells actively cross the blood/endothelium barrier to enter the tissue (50). BM homing of normal progenitor cells is dependent on CD44 expressed on these cells and HA displayed on the BM endothelium (51–53). Moreover, CD44 participates in homing and engraftment of various tumor cells (54–57).

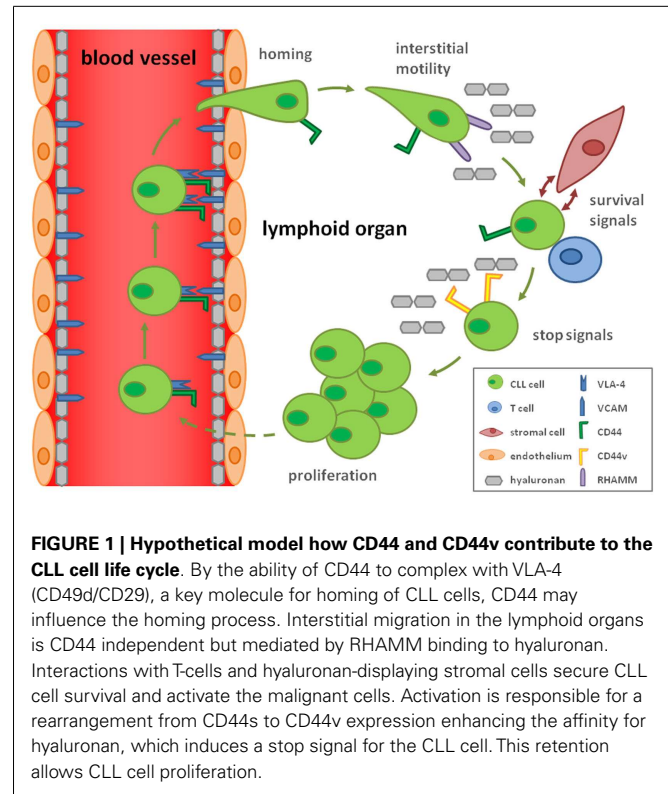
The contribution of CD44 to homing of CLL cells to BM and secondary lymphoid organs has not been dissected yet. However, we have previously established the integrin VLA-4, a heterodimer of the negative prognostic marker CD49d and the beta1 integrin subunit CD29, as the chief orchestrator of CLL BM homing (12, 58). Moreover, it was also shown that interaction of E-selectin with a specific glycoform of CD44 (HCELL) induces VCAM-1 binding of VLA-4. Thereby, HCELL ligation triggers inside-out upregulation of VLA-4 adhesiveness via G-protein dependent signal transduction leading to firm adhesion and subsequent transendothelial migration of human mesenchymal stem cells (59). Notably, in CLL, CD44v and VLA-4 constitute a cell surface docking complex for matrix metalloproteinase 9 (MMP-9) (in the pro and active form) (30). Here, proMMP-9 does not act as a protease upon docking to this surface receptor complex but fulfills functions in promoting CLL cell survival (60). MMP-9 lacks a transmembrane domain and is therefore dependent on cellular binding sites for all directed functions (61). Most recently, it was observed that high proMMP-9 expression and binding to these sites inhibits migration and reduces the homing capacity of CLL cells, suggesting a cooperation of VLA-4 and CD44(v) with MMP-9 (in the pro and active form) leading to CLL cell retention in lymphoid organs (62).

Consistent with this idea of CD44-mediated stop signals, we discovered that upon CLL cell activation by T-cells in lymphoid organs, high avidity CD44–HA interactions are formed due to induction of CD44v, most prominently CD44v6, harboring N-linked glycosylations. These interactions result in reduced cellular motility and lock CLL cells to immobilized HA. Thus, activation results in stop signals to migrating CLL cells by inducing strong cellular adhesion to the substrate, which may subsequently allow proliferation (29) (Figure 1). Since MMP-9 is particularly bound to CD44v rather than CD44 (30), it will be interesting how the suggested functions of MMP-9 in CLL are modulated by the activation-induced CD44v expression (29) and contribute to proliferation.

The suggestion of CD44(v) as a retention signal of interstitial motility – a process completely different from homing – is in line with early reports on the involvement of RHAMM rather than CD44 in IL-8-triggered motility of CLL cells on HA (63). This is consistent to our findings that blocking CD44 does not interfere with motility of resting CLL cells under shear free conditions but antagonizes their HA binding and adhesion, once they are activated (29).

### CD44 AND CLL CELL SURVIVAL

Human CD44 expression is increased by microenvironmental stimulation of CLL cells not only by CD40L-induced activation (29, 37) but also by the presence of feeder cells, known to provide prosurvival signals and early activation (64, 65). Activated CLL cells are protected against spontaneous and drug-induced apoptosis (66–68). Several previous studies suggested that CD44 is part of the survival signaling in CLL (37, 64, 69, 70). The addition of blocking anti-CD44 antibodies to CLL co-cultures with follicular dendritic cells reduced the survival of CLL cells, paralleled by decreased levels of the anti-apoptotic protein myeloid cell leukemia sequence 1 (Mcl-1) (64). Recently, Federochenko and



colleagues recapitulated the inhibitory effect of CD44 blockage or downregulation on Mcl1 protein levels *in vitro* and *in vivo*. To study the impact of CD44 on murine leukemogenesis, the authors used CD44 gene deletion, crossing CD44<sup>-/-</sup> animals with Eμ-TCL1 transgenic (tg) mice (37), which represent a well established murine model for CLL (71). In these mice, a CD5/CD19-double positive clonal B-cell hyperplasia arises in the peritoneal cavity and the disease subsequently spreads into other organs (spleen, BM, LNs, blood), with an overt leukemic phase starting from 8 to 10 months (71, 72). Eμ-TCL1 tg CD44<sup>-/-</sup> mice displayed a reduced peripheral blood tumor load at 12 months and significantly reduced spleen weights (37) compared to Eμ-TCL1 tg CD44<sup>+/+</sup> mice. The CD44 deficient murine CLL cells had marked signs of apoptosis, e.g., increased expression of cleaved caspase-3, suggesting a role of CD44 in tumor cell survival in the spleen microenvironment.

Notably, a novel humanized anti-CD44 mAb, RG7356, was recently found to induce apoptosis particularly in ZAP-70 positive CLL cells, in a caspase-dependent manner (70). The effects of this antibody occurred independent of complement and immune-effector cells and were attributed to ligation of CD44, altering its potential complexing with ZAP-70. This involvement of ZAP-70 in CD44-mediated CLL survival signaling and its physical complexing with CD44 clearly impacts on BCR signaling. ZAP-70 is known as an enhancer of BCR signaling upstream of survival and proliferation signals such as protein kinase B (Akt) and extracellular-signal-regulated kinases (ERKs) (73), which in turn induce anti-apoptotic proteins such as Mcl-1 and Bcl-xL (74). In consequence, the data may also suggest alterations of

the known complex of MMP-9 with CD44 and CD49d (VLA-4) (60) dependent on the BCR reactivity, which is shaped by ZAP-70.

## THERAPY

Therapeutically, CD44 is difficult to exploit due to its high variability and ability to complex with different partners in which CD44 function is apparently influenced. It is therefore not surprising that CD44 is not easily druggable, with some cases of previous failures (clinical trial identifier: NCT02254031; NCT02254044). The anti-CD44v6 antibody bivatuzumab (previously BIWA 4) coupled with a non-radioactive cytotoxic drug mertansine, for example, was used in studies against breast neoplasms (NCT02254005) and squamous cell carcinomas of the head and neck (HNSCC) (NCT02254018) (75–77). The death of one patient terminated the HNSCC trial (NCT02254044) whereas in the breast cancer study the antibody was found in non-tumor tissue as well and was therefore stopped (NCT02254031).

Nevertheless, several recent approaches could be advanced from preclinical status to testing in clinical trials. One promising candidate is the Å6 peptide (Ac-KPSSPPEE-amide), which is derived from the non-receptor binding domain of urokinase plasminogen activator and known to share a homologous sequence with CD44 (78, 79) (see also article by Finlayson in this volume). This homologous sequence (120-NASAPPEE-127) is found in the HA-binding site and is therefore present in all CD44 isoforms independent of alternative splicing events (80, 81). In preclinical studies, treatment with the Å6 peptide significantly decreased tumor growth and metastasis in a breast cancer mouse model without direct evidence of cytotoxicity or anti-proliferative activities toward the tumor (78). Instead, tumor and endothelial cell migration was clearly impaired by the peptide suggesting its impact on tumor invasion, metastasis, and angiogenesis. Similarly, Å6 reduced lymph node metastasis in a prostate cancer model (82). Notably, Å6 inhibited the migration of a subset of ovarian and breast cancer cell lines *in vitro* by inducing high adhesion of the CD44-expressing cells to an HA substrate and altering CD44 conformation (79), obviously locking the cells to substrates of HA, abundantly found, e.g., in LNs. First clinical trials demonstrated that Å6 was well tolerated (83, 84), resulting at least in an increased time to clinical disease progression of women with epithelial ovarian, fallopian tube, or primary peritoneal cancer in clinical remission (85) (NCT00083928). Currently a phase 2 trial is under way to determine the safety, tolerability, and efficacy of Å6 in CLL patients (NCT02046928).

A second promising candidate in CLL is the anti-CD44 antibody RG7356 (also known as RO5429083 or ARH460-16-2), a humanized antibody targeting a glycosylated, extracellular constant region of CD44 (86). As outlined above, this apoptosis-inducing antibody exerts a particular influence on BCR signaling in CLL and may be promising in light of the current success of all BCR-downstream-signal-targeting drugs.

Two clinical trials are underway to examine the pharmacokinetics, pharmacodynamics, safety, and efficacy of RG7356 in acute myelogenous leukemia (AML) patients and patients with metastatic and/or locally advanced CD44-expressing solid tumors.

## SUMMARY AND OPEN QUESTIONS

In summary, CD44 emerges as a key molecule of CLL cell interactions with the lymphoid microenvironment, shaping malignant cell positioning, and in consequence survival and proliferation in a fine-tuned manner. Nevertheless, some open questions remain on the mode of CD44 regulation in dependence of the activation status of the cells and the respective complex partner, such as CD49d/VLA-4. In addition, little is known on the second chief receptor interacting with HA, RHAMM. It is conceivable that RHAMM and CD44 fulfill distinct functions of cell migration and retention in CLL, which should be addressed in more detail in future. A deeper understanding of the functional regulation of CD44–HA interactions by splicing events and posttranslational modifications might help solving the existing controversies of its role in prognosis and survival. More functional studies as well as comprehensive patient cohorts and a clear clinical stratification of the patient groups would allow addressing these issues with sufficient statistical power and also assisting in the choice of the appropriate type of CD44 antagonizing therapy in CLL.

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