CD40-Mediated Activation of Chronic Lymphocytic Leukemia Cells Promotes Their CD44-Dependent Adhesion to Hyaluronan and Restrictions CCL21-Induced Motility

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Abstract
Microenvironmental interactions are crucial for the survival and proliferation of chronic lymphocytic leukemia (CLL) cells. CD4+ T cells that express CD40 ligand (CD40L), along with other accessory immune and stromal cells within CLL lymph nodes, provide signals needed for activation and outgrowth of the tumor clone. Furthermore, correct positioning of CLL cells within lymphoid subcompartments is essential for the transmission of these supportive signals. Thereby, interstitial cell migration and adhesion events, influenced by activational stimuli, determine CLL cell localization. CD44 has been implicated in cell activation, migration, and tissue retention via binding to its extracellular matrix ligand hyaluronan (HA). In this study, we investigated the role of CD44–HA interactions for CLL positioning and interaction with supportive microenvironments in peripheral lymph nodes, focusing on its regulation via CD40L-dependent, T-cell-mediated activation of CLL cells. We found that hyaluronan triggered a robust CCL21-induced motility of resting CLL cells. However, CD40L stimulation promoted the firm, CD44-mediated adhesion of CLL cells to hyaluronan, antagonizing their motile behavior. N-linked glycosylations of CD44, particularly associated with the variant isoform CD44v6 after CD40L activation, seemed to facilitate hyaluronan recognition by CD44. We propose that the CD40L–CD40 signaling axis provides a stop signal to motile CLL cells within lymph node compartments by inducing high avidity CD44–HA adhesion. This might retain CLL cells close to T-cell stimuli and facilitate essential interactions with hyaluronan-bearing stromal cells, collectively promoting CLL cell proliferation and survival. Cancer Res; 73(2); 561–70. ©2012 AACR.

Introduction
Chronic lymphocytic leukemia (CLL) is an incurable malignancy of mature B lymphocytes, which progressively accumulate in the peripheral blood, bone marrow, and lymph nodes. The interaction of CLL cells with accessory cells, the extracellular matrix, and soluble factors within the lymphoid microenvironment is essential for CLL cell proliferation and survival (1). CLL cells within lymphoid compartments display an activated CD69+ phenotype (2), and their proliferation occurs adjacent to CD40 ligand (CD40L) expressing CD4+ T cells and stromal cells within so-called proliferation centers (3, 4). This and further evidence (5, 6), led to the widely accepted concept that CD4+ T lymphocytes play an essential role in CLL cell activation, proliferation, and survival. CD40L, a TNF superfamily member expressed on activated T cells, has been described as a key mediator of T cell–driven CLL responses, acting in concert with T cell–derived cytokines (7–10).

Healthy T cell–dependent immune responses require the dynamically regulated positioning of B cells to specialized lymphoid subcompartments, which facilitate B-cell activation, proliferation, and differentiation (11). Activating stimuli alter the responsiveness of chemokine and adhesion receptors and thereby determine B-cell migration and retention. Lymph node chemokines coimmobilized to extracellular matrix molecules serve as the respective directional cues (12, 13).

In CLL, little is known about the stop and go signals involved in malignant B-cell positioning within the infiltrated lymph node microenvironments.
nodes, displaying a completely disrupted architecture. CD44 interactions with the extracellular matrix glucosaminoglycan hyaluronan (HA) participate in lymphocyte activation, migration, and tissue retention. The CD44 family (panCD44) comprises diverse isoforms, resulting from alternative splicing of 9 variant exons and extensive posttranslational glycosylations and glucosaminoglycan additions. Concomitantly, the hyaluronan-binding ability of the CD44 molecule is strictly controlled in a cell-type specific manner by CD44 clustering, CD44 variant isoform (CD44v; ref. 14) or glycoform expression (15, 16). In particular, leukocytes mainly express the standard CD44 isoform (CD44s), which lacks all variant exons, and require stimulation to recognize hyaluronan.

Correlative studies have suggested CD44 as a negative prognostic marker in CLL (17–19), but the underlying molecular mechanisms are not fully understood. Here, we aimed to examine the role of CD44–HA interactions for CLL cell positioning within lymph node compartments. We observed an activation-induced, CD44-mediated adhesion to hyaluronan, which was accompanied by increased expression of CD44v6 displaying N-linked glycosylations. We propose that the CD40L-dependent CLL cell activation within lymph nodes may provide a stop signal to motile CLL cells by inducing high avidity CD44–HA interactions, thus contributing to their retention required for survival and proliferation.

Materials and Methods

Patient samples
Following informed consent, blood samples were obtained from patients with CLL, which were chemonaive or had not received chemotherapy during the last 6 months, at the Third Medical Department Salzburg. Peripheral blood mononuclear cells (PBMC) were obtained by density gradient centrifugation, viable frozen and stored in liquid nitrogen. Thawed PBMCs were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) and antibiotics. For functional assays, CLL cells were enriched untouched using the EasySep Kit (Stem Cell Technologies). For RNA and protein isolation, CLL cells were positively isolated via CD19 microbeads (MACS, Miltenyi). Primary mesenchymal stromal cell cultures were established from bone marrow aspirates from healthy donors as described (20).

CLL cell activation
PBMCs from patients with CLL were cultured at a concentration of $2 \times 10^7$/mL for 15 hours on confluent layers of NIH/3T3 murine fibroblasts transfected with human CD40L or empty vectors (MOCK), kindly provided by Katja Zirlik (Department of Hematology and Oncology, University Medical Center, Freiburg, Germany). NIH/3T3 cells were periodically authenticated by morphologic inspection. CD40L expression on CD40L-transfected and control cells was constantly monitored by flow cytometry. For activation by autologous T cells, $2 \times 10^7$ PBMCs/mL were cultured on MOCK-transfected fibroblasts in the presence of human T-cell activator CD3/CD28 dynalbeads (Invitrogen) for 72 hours. Cells cultured without dynalbeads served as unstimulated controls.

Cell lines
Jurkat cells were obtained from the American Type Culture Collection (ATCC), where authentication was conducted by DNA fingerprinting with short tandem repeat (STR) profiling. After purchase, Jurkat cells were passaged fewer than 6 months. Lysates from HT29, HeLa, HepG2, and A431 cells, which were authenticated by ATCC by STR profiling, were purchased from Abcam. MCF-7 cells were kindly provided by Nadia Dandachi (Medical University Graz, Graz, Austria), passaged fewer than 3 months and used without further authentication.

Antibodies and reagents
Fluorochrome-labeled monoclonal antibodies (mAb) were purchased from BD or Beckmann Coulter. Anti-CD44v3 (clone VFF-327v3), anti-CD44v6 (clone VFF-18), and anti-panCD44 mAbs (clone SFF-304) were obtained from eBioscience. The blocking anti-CCR7 mAb (clone IM7 and 515) was purchased from BD. Human or rooster hyaluronan, porcine chondroitin sulfate B (CS), hyaluronidase (HAase) from Streptomyces hyalurolyticus, recombinant PNGase F and recombinant endoglycosidases F1 and F2 from Elizabethkingia miricola were obtained from Sigma-Aldrich. Fluorescein-conjugated hyaluronan (FL-HA) was kindly provided by Pauline Johnson (Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia, Canada).

Immunofluorescence
Immunofluorescence stains were conducted on formaldehyde-fixed, paraffin-embedded lymph node tissue samples of patients with CLL using polyclonal anti-CCL21 (R&D), hyaluronan-binding protein (biotinylated, Merck), anti-CD19 (AbD Serotec), anti-Ki-67 (eBioscience), and respective secondary antibodies. Samples were mounted in Prolong Gold antifade reagent with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen).

Cell motility
Microscopy chambers (Ibidi) were coated with 1 mg/mL hyaluronan or CS, followed by 4 µg/mL CCL21 and blocked with 2% human serum albumin (HSA). Hyaluronan was digested by 30 U/mL HAase for 1 hour at 37°C. For inhibition experiments, CLL cells were incubated with anti-CCR7 or anti-CD44 mAbs (10 µg/mL) or hyaluronan (100 µg/mL) for 15 minutes. Five minutes after cells were applied to motility chambers, noninteracting cells were washed away. The chambers were placed on a heated (37°C) stage of an inverted phase-contrast microscope (Olympus IX81) and at least 60 cells per field were recorded by time-lapse videomicroscopy using a 20-fold magnification. Cells moving more than 5 cell diameters during the observation time of 1 hour were considered motile. Motile cells and their velocities were determined by manual tracking of single cells using the "Chemotaxis and Migration" plugin of the ImageJ software.

Scanning electron microscopy
Fifteen minutes after being subjected to hyaluronan or HA/CCL21 substrates, CLL cells were fixed with 2.5%...
glutaraldehyde and dehydrated by ethanol and hexamethyldisilazane. Cells were glued on pins and sputter coated with a 61.2-nm gold layer, before the cell morphology was investigated with a Stereoscan 250 scanning electron microscope (Cambridge Instruments).

Flow cytometry
PBMCs were stained with mAbs specific for CD69, CD80, CD86, CCR7, panCD44, CD44v3, CD44v6, or corresponding isotype controls. To detect soluble hyaluronan (sHA) binding, PBMCs were incubated with FL-HA for 15 minutes at 4°C. CD44 was blocked by preincubation with 10 μg/mL anti-CD44 mAb (clone 515). N-linked glycosylations were removed by preincubation with endoglycosidase F1 and F2 solved in PBS and 2% FCS, according to the manufacturer’s guidelines. CLL cells were identified by anti-CD5 and anti-CD19 stainings. Viable cells were identified as Annexin V and 7-aminoactinomycin (7-AAD)—negative cells.

Adhesion assays
Cell culture–treated dishes were coated with hyaluronan (1 mg/mL) overnight, blocked with 2% HSA, and assembled as the lower wall of a parallel plate flow chamber (Glyco-tech). CLL cells were allowed to interact with the substrate for 1 hour at 37°C. CD44 was blocked by preincubation with 10 μg/mL anti-CD44 mAb (clone 515). N-linked glycosylations were removed by preincubation with endoglycosidase F1 and F2 solved in PBS and 2% FCS, according to the manufacturer’s guidelines. CLL cells were identified by anti-CD5 and anti-CD19 stainings. Viable cells were identified as Annexin V and 7-aminoactinomycin (7-AAD)—negative cells.

PCR analyses
Quantitative real-time PCR was conducted as described (21). For detection of CD44v transcription by reverse transcription PCR (RT-PCR), cDNA from CLL cells was amplified by panCD44 or CD44 variant exon specific primers as previously described (22) and visualized by 1% agarose ethidium bromide gels.

Immunoblotting
Immunoblotting under denaturing and reducing conditions was conducted as described (23) using mAbs specific for panCD44 (clone SFF-304), CD44v3 or CD44v6. For deglycosylation whole-cell lysates were digested by PNGase F according to the manufacturer’s directions.

Statistical analysis
Statistical analysis was conducted using GraphPad Prism 5.0. All data were tested for normal distribution by the Kolmogorov–Smirnov test. Normally distributed data were analyzed by paired t test, non-normally distributed by Wilcoxon matched pairs test. Differences were considered statistically significant when P < 0.05.

Results
Hyaluronan and CCL21 are colocalized within the reticular network of CLL lymph nodes and collectively trigger the robust motility of CLL cells
To identify which lymph node areas could facilitate CLL cell interactions with hyaluronan, we evaluated the hyaluronan distribution pattern in lymph node sections of patients with CLL. We found that hyaluronan was highly abundant throughout the infiltrated lymph nodes, including sites of low proliferation (Fig. 1A, left), as well as CLL cell proliferation centers, determined by the accumulation of Ki-67+ CLL cells (Fig. 1A, right). CLL cells were determined by CD19/CD5 costaining (data not shown). This suggests that resting as well as proliferating CLL cells are in close contact with hyaluronan.

To better understand the physiologic role of hyaluronan for CLL cells within lymph nodes, we established an in vitro system to determine the lateral migration of CLL cells on immobilized hyaluronan in the presence or absence of coimmobilized chemokines by time-lapse videomicroscopy. We observed that hyaluronan on its own failed to support the spontaneous migration of unactivated CLL cells. In contrast, surfaces displaying hyaluronan coimmobilized with CCL21 (herein HA/CCL21) induced CLL cells to migrate randomly with an average velocity of 5.0 ± 1.6 μm/min (Fig. 1B). CCL19 and CXCL13, 2 other chemokines contributing to B-cell positioning within the lymph nodes, induced a less robust migratory response (data not shown), wherefore we focused our studies on CCL21.

Scanning electron microscopy illustrated the round morphology of CLL cells positioned on hyaluronan, reflecting their nonmotile phenotype. However, CLL cells on HA/CCL21 gained a polarized morphology with highly branched membrane projections and large lamellipodia, indicating cell locomotion (Fig. 1C).

The CCL21-induced movement on hyaluronan was dependent on the chemokine receptor CCR7, as shown by blocking experiments (Fig. 1D). In addition, the presence of high molecular weight hyaluronan was indispensable, as the enzymatic treatment of HA/CCL21 coatings with HAase diminished cell motility. Consistently, CCL21 coimmobilized with CS, another glucosaminoglycan chemically closely related to hyaluronan, failed to support CLL cell locomotion (Fig. 1E). CD44 blockage with the mAb clone IM7 did not affect the cell motility (Fig. 1F), whereas blockage using a different clone (515) even enhanced the motile response (data not shown), suggesting that hyaluronan-mediated motility in CLL was triggered by a CD44-independent pathway(s). This is in line with previous observations describing HA/IL-8–induced motility of CLL cells being mediated by the receptor for hyaluronan-mediated motility (RHAMM; ref. 24).

To verify that CLL cells simultaneously encounter hyaluronan and CCL21 in vivo, we next assessed their relative distribution by fluorescence costainings. CCL21 was distributed throughout the infiltrated lymph nodes, and colocalized with hyaluronan at the reticular network, previously suggested...
to serve as guiding structure for leukocyte migration (Fig. 1G; ref. 25).

**CD40L stimulation of CLL cells induces an activated phenotype with reduced motility on immobilized HA/CCL21**

Next, we determined how CD40L stimulation, supposed to occur in lymph nodes, influences the CLL cell phenotype and migratory behavior on HA/CCL21. To mimic T-cell support, we stimulated peripheral blood–derived CLL cells *in vitro* by CD40L-transfected fibroblasts, or activated autologous T cells.

CLL cells cultured on CD40L-overexpressing fibroblasts gained an activated phenotype with strongly increased CD69, CD80, and CD86 surface levels as compared with cells cultured on control fibroblasts (Fig. 2A). Coculture with activated, autologous T cells induced a comparable activated CLL cell phenotype (Fig. 2B) followed by their proliferation (Supplementary Fig. S1).

Because the fibroblast-based CD40L activation system generated highly homogeneous activation profiles among all patient samples, we conducted functional studies with this system, but confirmed key findings on CLL cells stimulated with activated T cells.

By time-lapse videomicroscopy we investigated how activation modulates the migratory behavior of CLL cells on HA/CCL21. The motility of CD40L-stimulated cells was significantly decreased compared with unstimulated controls (Fig. 2C). CD40L-activated CLL cells expressed higher CCR7 levels than unstimulated controls (Fig. 2D) and fully retained their chemotactic response toward soluble CCL21 in transwell assays (Supplementary Fig. S2). Thus, the reduced migration of

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**Figure 1.** Hyaluronan (HA) and CCL21 are colocalized within the reticular network of CLL lymph nodes and collectively trigger the robust motility of CLL cells. **A,** fluorescence images, representative for 3 patients analyzed, show hyaluronan (green) and Ki-67 (orange) in combination with nuclear DAPI (white) stains of consecutive CLL lymph node sections. Scale bars, 50 μm. **B,** CLL cell motility on immobilized hyaluronan with or without CCL21 was analyzed by time-lapse videomicroscopy (n = 10). C, representative scanning electron images show CLL cell morphology on immobilized hyaluronan or HA/CCL21. Scale bars, 5 μm. **D,** CLL cells were pretreated with a blocking anti-CCR7 mAb before being subjected to immobilized HA/CCL21 (n = 6). **E,** cell motility on HA/CCL21 coatings treated with HAase and untreated coatings (n = 2). Where indicated, CLL cell motility on CS/CCL21 was compared with HA/CCL21 coatings (n = 5). **F,** CLL cells were pretreated with a blocking anti-CD44 mAb (n = 2). **G,** fluorescence images, representative for 3 patients, show hyaluronan and CCL21 costainings within the reticular network of a CLL lymph node section. Scale bar, 20 μm. *P* < 0.05; **P** < 0.01; ***P*** < 0.001.
CD40L-activated CLL cells on HA/CCL21 coatings did not result from a decreased CCL21 responsiveness, but rather from their altered interaction with hyaluronan. This prompted us to investigate a potential difference of hyaluronan-binding properties in unstimulated and CD40L-activated CLL cells.

**CD40L activation induces a strong hyaluronan binding in CLL cells, which is entirely CD44-mediated**

Cell migration requires an intermediate attachment strength between a cell and its substrate, enabling the generation of traction force to pull the cell forward but also permitting retraction of the cell rear (26). Therefore, we tested the binding of CLL cells to soluble FL-HA via flow cytometry. Although unstimulated CLL cells interacted with immobilized hyaluronan through weak interactions, which promoted their motility, these cells failed to bind soluble FL-HA, reflecting a rather weak affinity for hyaluronan. In contrast, FL-HA binding was observed in a considerable portion of CD40L-activated CLL cells and was entirely CD44-mediated as shown by blocking experiments (Fig. 3A). Similarly, CLL cells cocultured with activated T cells showed comparable levels of hyaluronan binding (Fig. 3B). Notably, this binding was also inducible by other stimuli, for example, phorbol 12-myristate 13-acetate (PMA)/ionomycin or immunoglobulin M (IgM)-cross-linking (Supplementary Fig. S3), suggesting that hyaluronan binding is a general phenomenon of CLL cell activation rather than an exclusive effect of CD40L stimulation.

To confirm that the high avidity CD44–HA interactions by themselves are responsible for the reduced motility of CD40L-activated CLL cells on HA/CCL21, we blocked these interactions by preincubation with saturating amounts of sHA. Indeed, this restored the motile phenotype of CD40L-stimulated CLL cells on the HA/CCL21 substrate (Fig. 4A). sHA treatment also reduced the adhesion of CD40L-activated CLL cells to immobilized hyaluronan confirming its blocking effect on CD44–HA bonds (Fig. 4B, left). Instead, cell rolling under shear force was increased (Fig. 4B, right), pointing to hyaluronan-dependent interactions of a lower strength. These results show that CD40L activation per se did not alter the migratory potential of CLL cells on HA/CCL21 but induced strong CD44-mediated adhesions to hyaluronan, which locked CLL cells to the substrate and thereby overruled the CCL21-mediated promigratory signal.

**CD40L-induced CD44–HA bonds “lock” CLL cells to hyaluronan and overrule CCL21-mediated promigratory signals**

To confirm that the high avidity CD44–HA interactions by themselves are responsible for the reduced motility of CD40L-activated CLL cells on HA/CCL21, we blocked these interactions by preincubation with saturating amounts of sHA. Indeed, this restored the motile phenotype of CD40L-stimulated CLL cells on the HA/CCL21 substrate (Fig. 4A). sHA treatment also reduced the adhesion of CD40L-activated CLL cells to immobilized hyaluronan confirming its blocking effect on CD44–HA bonds (Fig. 4B, left). Instead, cell rolling under shear force was increased (Fig. 4B, right), pointing to hyaluronan-dependent interactions of a lower strength. These results show that CD40L activation per se did not alter the migratory potential of CLL cells on HA/CCL21 but induced strong CD44-mediated adhesions to hyaluronan, which locked CLL cells to the substrate and thereby overruled the CCL21-mediated promigratory signal.

**CD40L stimulation elevates panCD44 levels and induces the expression of the variant isoforms CD44v3 and CD44v6**

The differential capacity of CD44 on unstimulated and CD40L-activated CLL cells to bind hyaluronan, led us to investigate potential alterations in their panCD44 levels or structural changes within the expressed CD44 repertoire. We
found a transcriptional upregulation of panCD44 upon CD40L activation (Fig. 5A), which translated into a significantly increased surface expression (Fig. 5B). However, all patients with CLL, irrespective of their clinical risk profile, uniformly displayed high panCD44 levels on peripheral blood CLL cells, which poorly bound hyaluronan (Supplementary Fig. S4). Therefore, the induction of hyaluronan binding only by increased panCD44 surface levels is improbable and we investigated CD44v expression of CLL cells.

CD44 encoded, mature mRNA of unstimulated CLL cells contained the variant exons v3, v5, v6, v7, v8, v9, and v10, as detected by RT-PCR. Particularly v3 and v6 were increased upon CD40L stimulation (Fig. 5C). Consistently, CD40L activation increased the surface expression of CD44 isoforms containing v3 and v6 (CD44v3 and CD44v6; Fig. 5D). A small population of CLL cells remained CD44v6-negative, indicating low activation level (Supplementary Fig. S5). CD44v6 expression and the hyaluronan-binding capacity was induced simultaneously in CLL cells cultured on CD40L-expressing fibroblasts (Fig. 5E). Confirmingly, CLL cell activation by activated T cells caused increased panCD44 and CD44v6.

Figure 3. CD40L activation induces a strong hyaluronan binding in CLL cells, which is entirely CD44-mediated. A, cytometrical analysis of soluble FL-HA binding to unstimulated or CD40L-stimulated CLL cells (n = 10, left). Where indicated, CLL cells were pretreated with a blocking anti-CD44 or isotype-matched control mAb (IgG1; n = 6, right). Results are illustrated in box plot format, whereby the median, the 25th, and 75th percentile and the minimum and maximum observations are depicted. B, cytometrical analysis of FL-HA binding of CLL cells after stimulation with activated T cells (n = 9). C, shear-resistant adhesion of unstimulated or CD40L-activated CLL cells on immobilized hyaluronan (n = 8, left). Where indicated, CLL cells were pretreated with a blocking anti-CD44 mAb or isotype control (IgG1; n = 6, right). /C3, P < 0.05; /C3/C3, P < 0.01; /C3/C3/C3, P < 0.001.

Figure 4. CD40L-induced CD44–HA bonds “lock” CLL cells to hyaluronan and overrule CCL21-mediated promigratory signals. A, CD40L-stimulated CLL cells were pretreated with sHA to block high avidity interactions with immobilized hyaluronan. The motility on HA/CCL21 was determined by videomicroscopy (n = 6). B, CD40L-stimulated CLL cells were left untreated or incubated with sHA before the analysis of shear-resistant adhesion (left) and rolling (right) on hyaluronan-coated plates or control plates lacking hyaluronan (n = 5). Bars, mean ± SD; *, P < 0.05; **, P < 0.01; ***, P < 0.001.
surface expression (Fig. 5F), whereas CD44v3 remained unaffected (data not shown).

**N-linked glycosylations, possibly linked to CD44v6, facilitate hyaluronan binding of CD44**

To detect if CD40L-triggered alterations in CD44 glycoform expression could facilitate its hyaluronan-binding capacity, we analyzed the molecular weight of CD44 by immunoblotting with or without removal of N-linked glycosylation by PNGase F. Using the panCD44 mAb, we found basal N-linked glycosylations of the predominant, approximately 90 kDa CD44 iso/glycoform, which were unaffected by the CLL activation status (Fig. 6A). CD40L stimulation induced the expression of CD44v6, with the high molecular weight of approximately 150 kDa, which was reduced after PNGase F treatment (Fig. 6B). Negative and positive controls proved the specificity of the used mAbs (Supplementary Fig. S6).

The removal of N-linked glycans by endoglycosidase F1 and F2 decreased FL-HA binding of CD40L-activated CLL cells (Fig. 6C). This shows that hyaluronan-binding properties of CD44 on CD40L-activated CLL cells are at least partially facilitated by N-linked glycosylations, found to be associated with CD44v6 but potentially also other isoforms.
CD40L activation potentiates the adhesion of CLL cells to primary stromal cells in a CD44-dependent manner

Stromal contact within the lymph node microenvironment is thought to support CLL outgrowth and survival. Therefore, we determined the importance of CD44-mediated adhesions to hyaluronan-bearing primary mesenchymal stromal cells. We found that CD40L stimulation highly increased CLL cell adhesion compared with unstimulated control cells (Fig. 7), which was largely CD44-mediated as shown by blocking experiments. An isotype matched control mAb had no effect (data not shown). The adhesion was also hyaluronan dependent, as HAase treatment of stromal cells decreased CLL cell adhesion (Fig. 7).

Discussion

CLL cells are known to interact with CD40L+ T cells within the lymphoid microenvironment, thereby receiving survival- and proliferation-inducing signals (4). To date, molecular cues determining CLL cell positioning and retention in proximity to the supportive signals remain poorly defined.

We found that CLL cells that were activated via the CD40–CD40L axis used hyaluronan as a substrate for CD44(v)-mediated adhesion. This was accompanied by induction of CD44v isoforms carrying N-linked glycosylations, altering the CD44 affinity to hyaluronan. These strong interactions inhibited the hyaluronan-dependent motility by locking CLL cells to immobilized hyaluronan and overruled CCL21-mediated promigratory signals. We suggest that unstimulated CLL cells use the reticular network, simultaneously presenting hyaluronan and CCL21, for their interstitial migration. Once CLL cells encounter T cells, they presumably get activated via CD40–CD40L signaling. This induces strong CD44(v)–HA bindings, causing CLL cells to stop migrating and instead tightly adhere to hyaluronan-bearing stromal cells. Importantly, hyaluronan and Ki-67 stainings of CLL lymph nodes verified strong hyaluronan expression at areas of high CLL cell proliferation. We propose that their CD44–HA–dependent adhesion facilitates cell division by retaining CLL cells in close proximity to CD4+ T cells, providing survival- and proliferation-inducing signals, for example, T cell–derived interleukins.

Tumor-driving CD44–HA interactions have been described in a variety of cancers (27). In CLL, elevated CD44 serum levels and high panCD44 as well as CD44v surface levels have been suggested as prognostic markers. While apparent consensus exists on the association of high CD44 serum levels with poor clinical outcome (17, 18, 28), the prognostic relevance of panCD44 and CD44v surface levels on circulating CLL cells remains arguable. Our data do not support a prognostic value of high CD44 levels on peripheral blood CLL cells. Instead, we suggest CD44v to be increased on CLL cells upon their activation by T cells in lymphoid organs preceding their proliferation. Increased CD44v expression on circulating CLL cells in patients with poor outcome (19) might thus reflect their activated phenotype. It is reasonable to assume that CD44 is shed from the CLL cell surface by, for example, matrix metalloproteinases (MMP; ref. 29) after proliferation to disrupt their anchorage.

Figure 7. CD40L activation potentiates the adhesion of CLL cells to primary stromal cells in a CD44-dependent manner. The adhesion of unstimulated or CD40L-stimulated CLL cells to primary mesenchymal stromal cells. Where indicated, cells were pretreated with a blocking anti-CD44 mAb or stromal cells were treated with HAase before CLL cells were applied (n = 8). **, P < 0.01.
on the hyaluronan substrate and to restore their interstitial motility. This shedding might account for the increased CD44 serum levels observed in CLL cases with high proliferative activity.

CD44(v)–HA interactions likely contribute to CLL cell survival. Multiple studies have implicated that direct cell contact with stromal cells rescues CLL cells from spontaneous and drug-induced apoptosis (30, 31). We observed that CD40L activation significantly elevated the CD44–HA–mediated adhesion of CLL cells to mesenchymal stromal cells. CD44 signaling has been suggested to activate prosurvival pathways via Mcl-1 (32, 33) or CD44v complexing with VLA-4 and MMP-9, facilitating prosurvival signals via the hemopoxin domain of MMP-9 in CLL (34, 35). However, we did not observe a survival benefit of CD40L-activated CLL cells plated on isolated hyaluronan (data not shown), suggesting that CD44 per se did not induce prosurvival signals upon engagement with hyaluronan, but instead established and/or maintained close contact to stromal cells, enhancing the transmission of stromal-derived survival signals.

Prognostic potential and microenvironmental relevance have also been attributed to RHAMM, a second hyaluronan receptor in CLL (36). RHAMM and CD44 can act as competing molecules or compensate each other about hyaluronan-mediated functions (37, 38). Previous observations in CLL suggested a clear RHAMM-dependency of IL-8/HA–induced CLL cell motility (24). As CD44 was not accountable for HA/chemokine–induced motility of resting CLL cells but mediated strong HA adhesion of CD40L-activated CLL cells, we suggest a well-regulated balance of RHAMM-HA and CD44–HA interactions in this disease.

Notably, we found that N-linked glycosylations support hyaluronan recognition by CD44 on activated CLL cells, in line with previous studies on melanoma cells (15). As CD40L activation did not affect glycosylations of the basal CD44 iso/glycoform but particularly induced the expression of N-glycosylated CD44v6, we suggest that hyaluronan recognition is mediated by variant isoforms and depends on their glycosylation patterns. While the exact mechanism remains elusive in this study, we assume that N-linked glycosylations trigger hyaluronan binding in CLL by altering CD44v charge and/or structure, or more indirectly, by facilitating CD44 clustering, which enables strong hyaluronan binding.

Collectively, our study provides strong evidence that microenvironmental interactions of activated CLL cells preceding their proliferation within lymph nodes are facilitated by CD44 (v)–HA interactions. Therefore, a disruption of these bonds could represent a promising therapeutic approach. The targeting of distinct CD44 isoforms/glycoforms, having a restricted expression pattern would allow specific targeting of CD44–HA adhesions and circumvent some of the previously observed side-effects raised by potential therapies targeting ubiquitous-ly expressed panCD44 (39). Taken together, we propose that the interference with CD44(v)-mediated adhesions to hyaluronan could release the tumor cells from their protective niches, prevent their proliferation, and eventually provide strong synergism with conventional therapies.

Disclosure of Potential Conflicts of Interest
L. Weiss has honoraria from speakers bureau of Mundipharma and Celgene. No potential conflicts of interest were disclosed by the other authors.

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CD40-Mediated Activation of Chronic Lymphocytic Leukemia Cells Promotes Their CD44-Dependent Adhesion to Hyaluronan and Restricts CCL21-Induced Motility

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