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The Contribution of Hematopoietic Stem / Progenitor Cell and Bone Marrow Stroma Cell Cd44v6 and Cd44v7 to Hematopoiesis

Rahul Singh, Prabhu Thirupathi and Margot Zöller*

Department of Tumor Cell Biology, University Hospital of Surgery, Heidelberg, Germany

Abstract

Hematopoietic stem/progenitor cells (HSPC) and leukemia-initiating cells (LIC) require CD44 for homing and survival. As a first step to selectively attack LIC, we explored the engagement of standard versus CD44v6 and CD44v7 variant isoforms (CD44s, CD44v) in HSPC maintenance by antibody blocking, using CD44v7- and CD44v6/v7-knockout (^{ko}) mice.

HSPC matrix protein adhesion is dominated by CD44s. CD44v6 supports migration towards hyaluronan (HA), fibronectin, IL6, OPN, SDF1 and bone marrow stroma cells (BM-StrC), where BM-StrC CD44v7 strongly facilitates HSPC homing. Mitigated adhesion affects quiescence and drug resistance. CD44v6/v7^{ko} HSPC divide more frequently than wild type (wt) HSPC and anti-CD44v6 drives HSPC into proliferation. Apoptosis resistance is supported by HA and BM-StrC and is affected in CD4v6/v7^{ko} HSPC. HA and BM-StrC promote apoptosis resistance via PI3K/Akt pathway activation, which is dampened in CD44v6/v7^{ko} HSPC.

Thus, HSPC CD44 contributes to adhesion, migration, quiescence and apoptosis resistance. BM-StrC CD44v7 supports HSPC homing. HA- and BM-StrC-promoted quiescence and apoptosis resistance proceeds via HSPC CD44v6. As HSPC matrix adhesion mostly relies on CD44s, HSPC CD44v6/CD44v7 expression is low and CD44v7 influences the crosstalk with BM-StrC, attacking CD44v6-overexpressing LIC by anti-CD44v6 may not severely affect the HSPC interaction with the osteogenic niche.

Keywords: CD44 variant isoforms; Hematopoietic stem/progenitor cells; Bone marrow stroma; CD44 ligands

Abbreviations: BM: Bone Marrow; BMC: BM Cells; BM-Str: Bone Marrow Stroma; BM-StrC: BM-Str cells; HSPC: Hematopoietic Stem/Progenitor Cells; CD44s: CD44 standard isoform; CD44v: CD44 variant isoforms; FN: Fibronectin; HA: Hyaluronic Acid; ko: Knockout; LIC: Leukemia-Initiating Cells; OPN: Osteopontin; wt: Wild Type

Introduction

Leukemia initiating cells (LIC) share several features with hematopoietic stem / progenitor cells (HSPC), including the requirement for a homing and survival supporting niche [1]. Therefore, great efforts are undertaken to unravel the crosstalk between HSPC and the niche [2,3], where CD44 attracted considerable interest as a HSPC and LIC biomarker [4-6]. Taking this into account, it becomes demanding to define the contribution of hematopoietic cell- and niche-associated CD44 activities as well as the contribution of CD44 variant isoforms (CD44v) that might offer a possibility to spare HSPC while attacking LIC [7].

The adhesion molecule CD44, a glycoprotein varying in size due to N- and O-glycosylation and insertion of alternatively spliced variable exon products [8-10], is involved in a multitude of functions. CD44 is the major hyaluronan (HA) receptor [11,12], but also binds fibronectin (FN) [13]. HA binding is mediated by the CD44 standard isoform (CD44s), which does not contain variant exon products. Nonetheless, the binding affinity can be modulated by variant exon products and/or glycosylation, which is important for cell migration on HA [12,14]. In migrating cells, CD44 is guided to the leading edge by the interaction of the CD44 cytoplasmic tail with the cytoskeleton [15]. The CD44 linkage to the actin cytoskeleton proceeds via ankyrin and members of the ERM (Ezrin/Radixin/Moesin) family, where phosphorylated ERM proteins bind CD44 as well as actin [16-18]. CD44, partly via glycosaminoglycan binding sites, also is engaged in cytokine and chemokine binding, besides others bFGF, osteopontin and hepatocyte growth factor (HGF) [12,19,20]. The harboring feature of CD44 has significant functional consequences, e.g. osteopontin binding leads to signals that stimulate PI3-kinase and Akt activation with upregulation of anti-apoptotic proteins promoting bone marrow cell (BMC) survival [21]; HGF binding provides the initiating signal for c-Met autophosphorylation and activation [22]. CD44 also associates with the receptor tyrosine kinases EGFR, ERBB2, VEGFR2, TGFβRI and II [23-25]. Finally, CD44 acts as a receptor for cellular ligands such as CD62L [26].

In hematopoiesis CD44 is essential for the crosstalk with the osteogenic niche, which is required for HSPC maintenance [27]. Thus, anti-CD44 prohibits the development of cobblestone areas, clusters of HSPC, in murine long term bone marrow (LTBM) cultures [28]. BM stroma (BM-Str) formation also requires CD44 that supports the process by induction of IL-6 secretion [29]. HSPC synthesize and express HA and HA expression correlates with selective migration of HSPC to the endosteal niche. The essential role of the HSPC CD44 – HA interaction was demonstrated by blocking HSPC homing by anti-CD44, soluble HA or hyaluronidase treatment [30]. Furthermore, stroma-derived factor 1 (SDF1, CXCL12) stimulates adhesion of progenitor cells via CD44, which suggests HA and CD44 to be involved in SDF1-dependent transendothelial migration of HSPC and anchoring within

***Corresponding author:** Margot Zoller, Department of Tumor Biology, University Hospital of Surgery, Germany, Tel: 49 6221 565146; Fax 49 6221 565199; E-mail: m.zoeller@uni-hd.de

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the osteogenic niche [31]. Besides contributing to HSPC homing and settlement [32,33], CD44 promotes quiescence versus differentiation, which depends on associated integrins, proteases and chemokine ligands [34,35].

CD44 also accounts for LIC homing [27]. In acute myeloid leukemia (AML) CD44 is required for settlement in the osteogenic niche and anti-CD44 induces AML stem cell differentiation [36]. In a mouse model of chronic myeloid leukemia, BCR-ABL1-transduced progenitors from CD44-mutant donors were defective in BM homing, which resulted in impaired CML-like disease induction [37]. Our studies confirmed that leukemia cell homing and growth is retarded by a CD44-specific antibody, but also pointed towards an antibody blockade of CD44 during reconstitution severely affecting HSPC embedding [32,38,39].

To circumvent such potential drawbacks, we elaborated the contribution of HSPC and BM stroma cell (BM-StrC) CD44s, CD44v6 and CD44v7 to HSPC adhesion, homing, quiescence and apoptosis resistance. We demonstrate a dominant role of HSPC CD44s for adhesion. HSPC CD44v6 contributes to cytokine capture, migration and apoptosis resistance. BM-StrC CD44v7 facilitates HSPC adhesion and homing. Though the CD44v6-mediated activities support HSPC, they are not essential for HSPC maintenance. Thus a blockade of CD44v6 in leukemic cells should not severely affect hematopoiesis.

Material and Methods

Mice: BALB/c (H-2^d), SVEV (H-2^b), CD44v7^{ko} and CD44v6/7^{ko} (back-crossed to SVEV) mice [33,38], kindly provided by U. Gunthert, Department of Microbiology, University of Basel, Basel, Switzerland, were bred at the central animal facilities of the University of Heidelberg; 6-10wk old mice were used for experiments. Animal experiments were approved by the Government of Baden-Wuerttemberg, Germany.

Antibodies are listed in Suppl. Table 1. It should be mentioned that the anti-CD44 antibody IM7 binds an epitope of the CD44s molecule. Thus, it recognizes all CD44 isoforms and therefore is termed anti-panCD44.

RT-PCR

Total RNA was extracted using TRIzol reagent. Primers and RT-PCR conditions are listed in Suppl. Table 2.

Recombinant CD44 proteins (rCD44)

cDNA sequences of the extracellular domain of CD44s, CD44v6 and CD44v7 cDNA were obtained by PCR amplification and were inserted into The pCEP4 vector modified by insertion of a myc and his tag. HEK-293 EBNA cells were transfected using Polyfect following manufacturer's instructions (Qiagen, Hilden, Germany). Cells were cultured in ISCOVE/10% fetal calf serum (FCS)/L-glutamine/0.5 µg puromycin. rCD44 was purified from culture supernatant by passage over an anti-myc Sepharose column and elution with 0.2 M glycine buffer, pH 2.7. Eluted fractions were concentrated and dialyzed against PBS (Vivaspin tubes, 50 kDa cut off). Protein concentration was measured by Biorad assay.

Long term bone marrow stroma cells (BM-StrC)

BMC were collected from femora and tibiae of 6-10 wk old mice by flushing the bones with PBS through a 17gauge needle. Cells were washed and seeded in 50 ml cell culture flasks at a density of 2×10^6 cells/ml ISCOVE/20% horse serum/25 µM 2-ME/2 mM L-glutamine/10 µM hydrocortisone. Non- and loosely adherent cells were removed weekly by vigorous pipetting. When cultures reached confluence, the cells were detached with a rubber policeman and cells were split 1 in 2. From thereon, cells were maintained in ISCOVE/10%FCS/2 mM L-glutamine. Medium was exchanged weekly after vigorously washing off loosely attached cells. After 6 wk the monolayer of BM-StrC was detached with a rubber policeman and was used for further analysis.

HSPC isolation

BMC were stepwise depleted of $CD4^+$, $CD8^+$ and NK cells, $CD19^+$, $CD45^+$, $CD11c^+$ and $CD11b^+$, $Ter119^+$ and $Ly6C/G^+$ cells by magnetic beads coated with the respective antibodies (Miltenyi Biotec, Bergisch Gladbach, Germany). The depleted cell population was incubated with anti-CD117-coated beads collecting the adherent fraction. The $CD117^+$ cells were further enriched for HSPC by incubation with biotinylated anti-SCA1 and anti-biotin coated beads, collecting the adherent population. The regain of $CD117^+$ cells varied between 1.6% - 3.5% (mean: 2.5%). In BALB/C mice, roughly 30% of $CD117^+$ cells were $SCA1^+$, in SVEV the recovery of $SCA1^+$ cells from $CD117^+$ cells varied from 87%-100%. Viability of the separated populations was >98%.

Flow cytometry

Cells ($1\text{-}2.5\times10^5$) were stained according to routine procedures. For intracellular staining (cytokines, chemokines, signaling molecules) cells were fixed and permeabilized in advance. Apoptosis was determined by annexinV-FITC or -APC / PI staining. The cell cycle was controlled by PI staining (1 µg/ml, 0.1% TritonX-100, RNAase A) after fixation in ethanol. Samples were processed in a FACS-Calibur using the Cell Quest program for analysis (BD, Heidelberg, Germany).

Cells lysis, immunoprecipitation (IP) and Western blot (WB)

Cells (10^7) were washed twice in TNE-buffer (20mM Tris-HCl pH7.4, 150 mM NaCl, 2 mM EDTA, 1 mM Na_2VO_4, 10 mM NaF) and lysed in the same buffer containing 1% Brij98 or 1% TritonX-100, 1 mM PMSF and a protease inhibitor mix (Boehringer, Mannheim). After 30 min at 4°C, lysates were centrifuged (13000 g, 10 min, 4°C). Lysates were either analyzed by SDS-PAGE and WB or were used for IP. For IP, precleared lysates (1/10 volume ProteinG Sepharose, 1 h, 4°C) were incubated with the primary antibody (1 h/4°C) followed by incubation with ProteinG Sepharose (1 h/4°C). Immune complexes were washed 4 times with lysis buffer. Immunoprecipitated proteins were analyzed by SDS-PAGE, followed by WB. Lysates were resolved on 10% SDS-PAGE. Proteins were transferred to Nitrocellulose membranes and detected by WB with HRPO-conjugated antibodies or streptavidin using the ECL detection system.

Pulldown assay

Cell lysates were passed over rCD44 Sepharose columns. Bound proteins were eluted with 0.2 M glycine buffer, pH 2.7. The concentrated and dialyzed eluate was separated by SDS-PAGE for MALDO-TOF analysis.

MALDI-TOF analysis

After SDS-PAGE, gels were silver-stained. Protein digestion, sample preparation, MALDI-TOF fingerprint analysis, post-source decay fragmentation analysis and database searches were performed as described [40].

Adhesion assay

HSPC or BM-StrC were labeled with Carboxyfluorescein succinimidyl ester (CFSE) (10 µM, 1 h, 37°C). After centrifugation, cells were resuspended in RPMI1640/10% FCS (30 min, 37°C) to capture

free CFSE and to guarantee integration of uptaken CFSE. After washing, cells were seeded on plates coated with matrix proteins or cytokines or were seeded on a cell monolayer. Where indicated HSPC or BM-StrC were preincubated with anti-panCD44, anti-CD44v6 or anti-CD44v7 (10 µg/ml). For cell-cell adhesion, CFSE-labeled HSPC were seeded on BM-StrC. Cells were incubated for 4h at 37°C. After vigorous washing, adherent cells were lysed and fluorescence was evaluated in a fluorescence ELISA reader. The percentage of adherent cells is shown. The total input of cells was taken as 100%. Adhesion of recombinant proteins was evaluated by standard ELISA procedures. After binding of rCD44 to matrix protein- and cytokine-coated plates, AP-labeled anti-CD44 was added. After washing and incubation with substrate, optical density (OD) was measured at 450 nm.

Migration assays

Cells in the upper part of a Boyden chamber (RPMI/0.1%BSA) were separated from the lower part (RPMI/20%FCS) by 5 µm pore size polycarbonate-membranes. After 6h, cells in the lower chamber were counted. Migration is presented as percent of input cells. For video microscopy, unlabeled or CFSE-labeled cells (5×10^4) were seeded on a cell monolayer or on matrix protein-coated 24-well plates. Plates were placed under an Olympus IX81 inverse microscope with an Hg/Xe lamp, an incubation chamber (37°C, 5%CO_2), a CCD camera (Hamamatsu) and a ScanR acquisition soft ware (Olympus, Hamburg, Germany). Two pictures (20-fold magnification) / chamber (2 ms exposure) were taken every 20 min for 12 h. Migration was quantified according to Manual tracking plugin running in the open-source software Image J for 20 cells per well.

Proliferation, cell cycle and apoptosis

CFSE-labeled HSPC were cultured on BSA (1%) or HA (100 µg/ml) coated plates or on a BM-StrC monolayer. HSPC division was evaluated by flow-cytometry after 24 h-72 h. Cell cycle progression was evaluated by PI staining. For apoptosis induction (AnnV/PI staining), cells were cultured in the presence of cisplatin. Mean values ± SD of triplicates are presented.

Statistics

Significance of differences was calculated by the two tailed students T-test. If not stated otherwise, mean ± SD are derived from 3-5 experiments and/or are based on 3-4 replicates. P-values <0.05 were considered statistically significant.

Results

CD44 is required for HSPC homing, maintenance and retention in the osteogenic niche with a suggested contribution of HSPC and BM-StrC CD44. The contribution of BM-StrC CD44 is poorly defined. It is also unknown for both HSPC and BM-StrC, whether the CD44 standard (CD44s) or CD44 variant isoforms (CD44v) are engaged in these processes. To answer these questions, we characterized HSPC and BM-StrC CD44 expression, defined the most prominent ligands and evaluated the impact of HSPC and BM-StrC CD44s, CD44v6 and CD44v7 expression on adhesion, migration, quiescence and apoptosis resistance to obtain hints for attacking LIC, while largely sparing hematopoiesis.

CD44 expression in HSPC and BM-StrC

HSPC ($CD117^+/SCA1^+$) were enriched from BMC by magnetic bead sorting. CD44 / CD44v expression in $CD117^+/SCA1^-$ and $CD117^+/SCA1^+$ HSPC was evaluated by semiquantitative RT-PCR, with reverse primers in the CD44s exon S6 region and forward primers in the respective CD44v exon (Suppl.Table 2) such that e.g. amplifying exon v8 revealed potential bands for exon v8 or v8 and v9 or v8, v9 and v10 or v8 and v10 (Suppl. Figure 1).

Unseparated BMC, $CD117^+/SCA1^-$ and $CD117^+/SCA1^+$ cells showed besides CD44s expression, comparably strong CD44v7 and CD44v10 expression. In BMC it was mostly found in combination with CD44v8 and CD44v9, in $CD117^+SCA1^-$ cells in combination with CD44v9 and in $CD117^+SCA1^+$ cells as single exon (Figure 1A). Flow-cytometry confirmed panCD44, CD44v3, CD44v6, CD44v7 and CD44v10 protein expression in HSPC (Figure 1B). Besides CD117 and SCA-1, the majority of HSPC expressed the hematopoietic lineage markers CD34, FcγRIII (CD16), CD43, CD31, CD24 and CD71. They did not express markers of committed progenitors and of mature T cells and B cells (Figure 1C). A high percentage of HSPC also expressed the adhesion molecules CD49d, CD49e, CD49f, CD54 and CD102 (Figure 1D). Expectedly, expression of HSPC markers and progenitor markers differed in committed progenitors and mature leukocytes in the BM. However, with the exception of CD44v3 and CD44v10 in a mixed B cell / macrophage (Mφ) population, expression of CD44v3, -v6, -v7 and -v10 was very low or not detectable in T cells/NK, and committed erythroid/myeloid progenitors (Suppl. Figure 2A). Instead >50% of the latter population expresses Ter119 and Ly6G. Notably, too, the BM contained significantly fewer $CD4^+$ than $CD8^+$ cells. Mature leukocytes and committed progenitor cells in the BM frequently expressed CD16, CD43, CD31, CD71 and CD45R. Expression of CD117, SCA1 and CD34 was very low in committed progenitors and mature leukocytes in the BM (Suppl. Figure 2B).

HSPC homing, motility, quiescence and differentiation being strongly influenced by cytokines and chemokines in the niche, we finally evaluated cytokine / chemokine and receptor expression in unseparated BMC and HSPC. Over 30% of HSPC expressed the cytokines IL6 and OPN and the cytokine/chemokine receptors CD123, CD126, Flt3 and CXCR4. Expression of GM-CSF, IL3, IL7, thrombospondin (TSP), bone morphogenetic protein 4 (BMP4) and SDF1 was low to very low. Expression of OPN, CD123, CD126, CD127 and Flt3 was higher in HSPC than in unseparated BMC (Figure 1E).

For comparison, CD44v expression was also evaluated in BM-StrC. The mRNA expression profile in BM-StrC differed from that of HSPC inasmuch as multiple exon combinations were more abundant (Figure 1F). Flow-cytometry confirmed panCD44, CD44v3, CD44v6, CD44v7 and CD44v10 protein expression in BM-StrC, with higher expression levels of CD44v7 and CD44v10 in BM-StrC than HSPC (Figure 1G). BM-StrC did not express HSPC, progenitor or mature leukocyte markers (Figure 1H), but expressed the adhesion molecules CD49d, CD49e, CD54, CD51, CD61, CD105 and selectins, though mostly at a lower frequency than HSPC (Figure 1I). The cytokines / chemokines GMCSF, IL6, OPN, BMP4 and SDF1 were expressed at a high level in BM-StrC (Figure 1J).

Taken together, HSPC and BM-StrC share high expression of CD44. HSPC differ from BM-StrC in CD44v expression. HSPC express CD44v6 (weakly), CD44v7 and CD44v10; BM-StrC express a larger variety of CD44v isoforms and CD44v7 and CD44v10 at a higher level than HSPC. Thus, the question arose, whether the comparably weak expression of CD44v6 and CD44v7 on HSPC affects hematopoiesis.

The importance of CD44 expression for HSPC adhesion

Most CD44 activities depend on and are initiated by HA binding [4]. Furthermore, HSPC secrete HA, which is required for HSPC

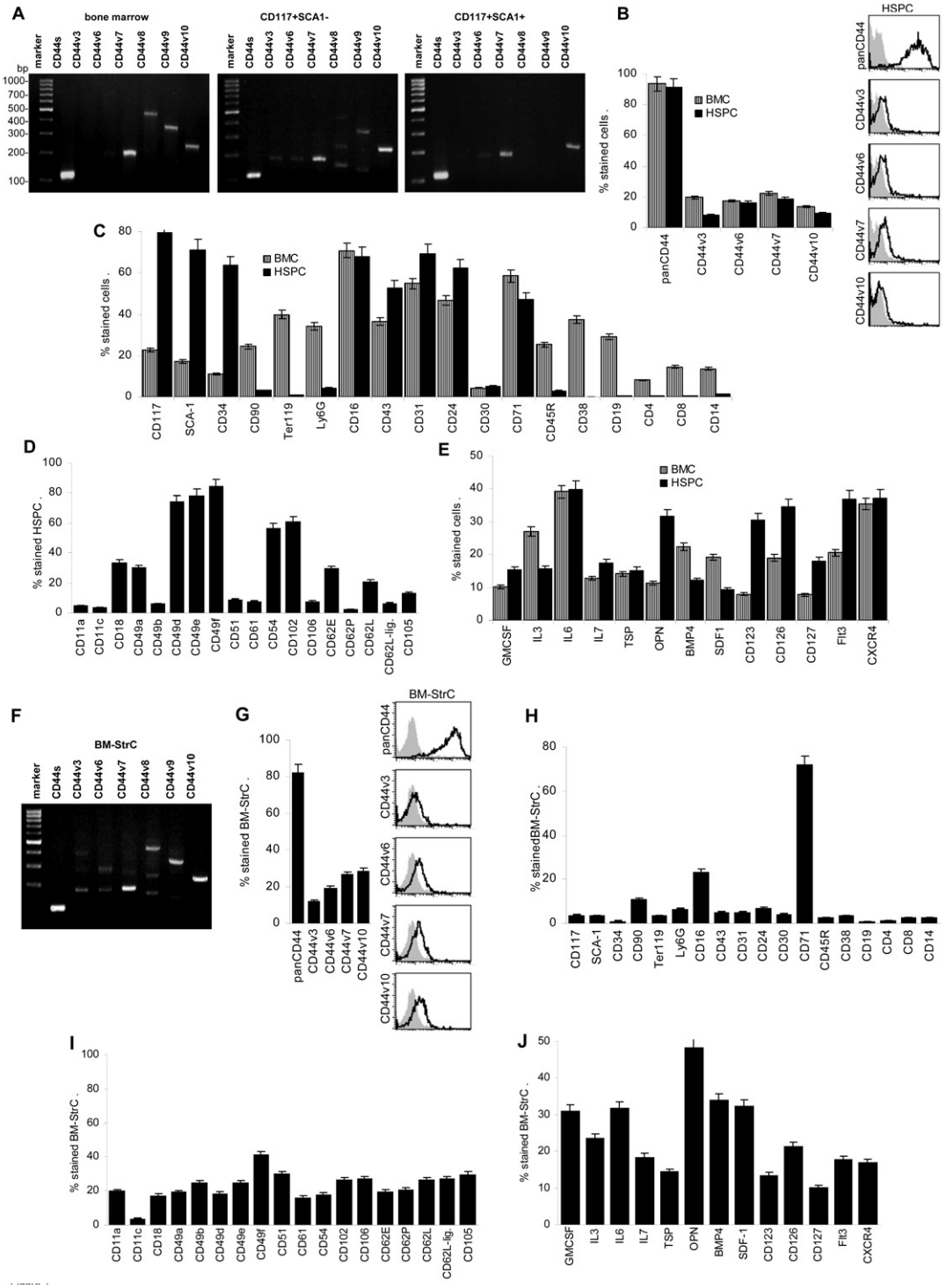

Figure 1: CD44 expression in BMC and BM-StrC: (A) Unseparated BMC, CD117$^+$SCA1$^-$, CD117$^+$SCA1$^+$ and (F) BM-StrC were analyzed for CD44 and CD44v expression by semiquantitative RT-PCR; (B) expression of panCD44, CD44v3, CD44v6, CD44v7 and CD44v10 was evaluated by flow cytometry in unseparated BMC and HSPC and (G) BM-StrC. Mean values ± SD and representative overlays are shown; (C) stem cell, progenitor and mature leukocyte marker expression were evaluated by flow cytometry in BMC and HSPC as well as (H) in BM-StrC; (D,I) adhesion molecule expression in HSPC and BM-StrC and (E,J) cytokine / chemokine and receptor expression in BMC, HSPC and BM-StrC. (B-E, G-J) The percent stained cells (mean ± SD, 3 assays) are shown.

CD44 is highly expressed in unseparated BMC and HSPC. HSPC mostly express single CD44v variant exon products at a low level. Expression of stem cell markers is highly enriched in the CD117$^+$SCA1$^+$ subpopulations, which does not express committed progenitor or mature leukocyte markers. Besides CD44, expression of the adhesion molecules CD49d, CD49e, CD49f, CD54 and CD102 is high in HSPC. In comparison to unseparated BMC, only expression of the chemokine OPN and the cytokine/ chemokine receptors CD123, CD126, CD127 and Flt3 are increased in HSPC. BM-StrC differ from HSPC by frequently expressing several CD44v exon combinations, with high expression particularly of CD44v7 and CD44v10. BM-StrC do not express HSPC, committed progenitor or mature leukocyte markers. Expression of adhesion molecules is mostly lower than in HSPC, but expression of chemokines / cytokines frequently exceeds expression in HSPC.

homing in the osteogenic niche [41]. Thus, we first evaluated the impact of CD44 / CD44v on matrix adhesion.

HSPC readily adhered to HA-, FN- and coll IV-coated plates, adhesion exceeding that of unseparated BMC. Adhesion of CD44v7^{ko} and CD44v6/v7^{ko} HSPC to HA was slightly impaired (Figure 2A). To control for the contribution of CD44v6 and CD44v7 to matrix protein adhesion, wt BMC and HSPC were preincubated with anti-panCD44, anti-CD44v6 or anti-CD44v7. All three antibodies inhibited HSPC

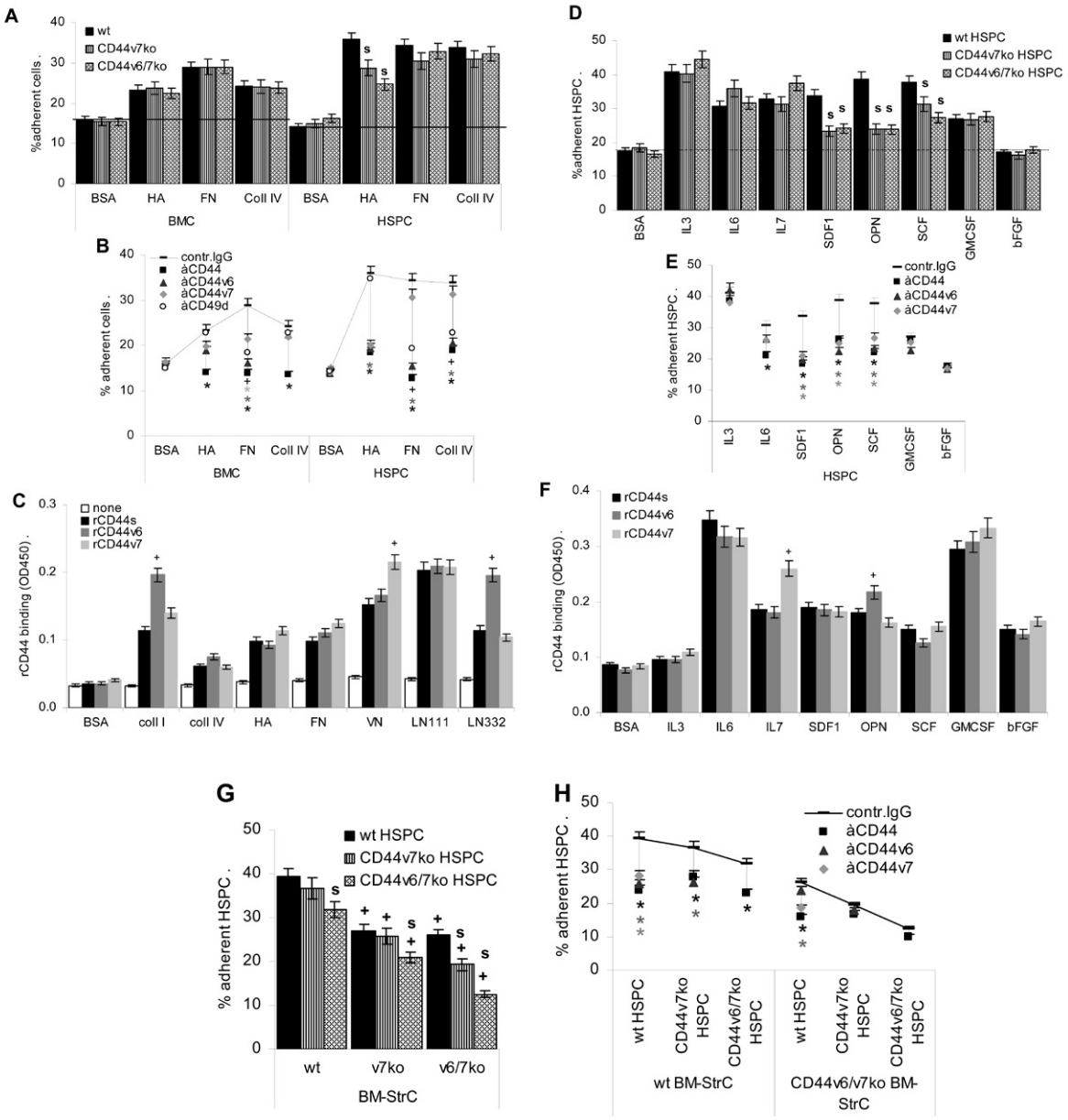

Figure 2: BMC, HSPC and BM-StrC adhesion: (A) CFSE-labeled BMC and HSPC from wt, CD44v7^{ko} and CD44v6/v7^{ko} mice were seeded on BSA, HA, FN or coll IV coated plates; (B) wt BMC and HSPC were preincubated with anti-panCD44, -CD44v6, -CD44v7 and -CD49d and were seeded on BSA, HA, FN or coll IV coated plates; (C) rCD44, rCD44v6 and rCD44v7 were seeded on BSA, HA, FN, coll I, coll IV, LN111 and LN332 coated plates; (D) wt, CD44v6/v7^{ko} and CD44v7^{ko} HSPC were seeded on cytokine or chemokine-coated plates; (E) wt HSPC were preincubated with anti-panCD44, -CD44v6 and -CD44v7 and were seeded on cytokine or chemokine-coated plates; (F) rCD44, rCD44v6 and rCD44v7 were seeded on cytokine or chemokine-coated plates; (G) CFSE-labeled wt, CD44v6/v7^{ko} and CD44v7^{ko} HSPC were seeded on a monolayer of wt, CD44v6/v7^{ko} and CD44v7^{ko} BM-StrC; (H) CFSE-labeled wt, CD44v6/v7^{ko} and CD44v7^{ko} HSPC were preincubated with anti-pan-CD44, -CD44v6 or -CD44v7 and seeded on a monolayer of wt or CD44v7^{ko} BM-StrC (A-H) Adhesion was evaluated after 4h incubation at 37°C, 5%CO_2; (A,B,D,E,G,H) after washing, adherent cells were lysed and fluorescence was evaluated in a fluorescence ELISA reader. Adhesion is presented as % of input cells; (C,F) Plates were washed, incubated with anti-CD44-AP and substrate. OD was determined at 450nm. All assays were performed in triplicates, mean ± SD are presented; (A,D,G): significant differences between wt and CD44v7^{ko} or CD44v6/v7^{ko} cells: **s**; (B,E,H) significant inhibition by anti-CD44: *, significant inhibition by anti-CD49d: +; (C,F) significant differences between rCD44s versus rCD44v6 or rCD44v7 binding: +; (G) significant differences in HSPC binding to wt versus CD44v7^{ko} or CD44v6/v7^{ko} BM-StrC: +.

HSPC CD44 contributes to HA, FN and less pronounced, coll IV adhesion; CD44v6/CD44v7 strengthen HA and FN adhesion. CD44 also binds cytokines / chemokines; CD44v6 and CD44v7 contribute to SDF1, OPN and SCF binding. Importantly, BM-StrC CD44v6/v7 also contributes to HSPC binding.

adhesion to HA and anti-panCD44 and anti-CD44v6, but not anti-CD44v7 also inhibited HSPC adhesion to FN and coll IV. As adhesion to FN and coll IV was not affected in CD44v7^{ko} and CD44v6/v7^{ko} HSPC, it is likely that inhibition was due to steric hindrance of CD44-associated adhesion molecules, like e.g. CD49d [42], which was controlled by repeating the experiment in the presence of anti-CD49d. Anti-CD49d had a minor impact on adhesion to HA, but inhibited adhesion to FN and coll IV (Figure 2B). Finally, the finding that binding of recombinant (r) CD44s, rCD44v6 and rCD44v7 (Suppl. Figure 3A) to HA-, FN- and coll IV-coated plates, though significantly exceeding binding to BSA-coated plates, did not strongly differ, is in line with mostly CD44s accounting for matrix protein binding. Nonetheless, rCD44v7 showed slightly enhanced binding to vitronectin (VN) and rCD44v6 to coll I and laminin (LN) 332. Of note, rCD44 efficiently binds VN and LN111 (Figure 2C).

CD44 also binds, directly or via associated molecules, chemokines and cytokines. CD44^{wt} HSPC bound to IL3, IL6, IL7, SDF1, OPN, SCF and weakly to GMCSF; they did not bind to bFGF. CD44v7^{ko} and CD4v6/v7^{ko} HSPC binding to SDF1, OPN and SCF was reduced compared to wt. HSPC binding (Figure 2D). Binding studies with wt HSPC preincubated with anti-panCD44 revealed that HSPC binding to IL3 and GMCSF was independent of CD44 expression, whereas binding of anti-CD44 pretreated HSPC to IL6, SDF1, OPN and SCF was reduced, though not abolished. Binding of HSPC to SDF1, OPN and SCF was also reduced by antibody blocking of CD44v6 or CD44v7 (Figure 2E). rCD44 confirmed IL6, IL7, SDF1, OPN and weak SCF binding. Distinct to HSPC, rCD44 also bound GMCSF and weakly bFGF. There was a tendency towards stronger binding of rCD44v7 to IL7 and of rCD44v6 to OPN, but differences were borderline ($p < 0.05$) statistically significant (Figure 2F).

Besides interacting with matrix proteins and soluble factors, HSPC bind to cellular components of the niche. In the search for cellular ligands, HSPC and BM-StrC were lysed using mild lysis conditions not to destroy protein complexes. Lysates were passaged over Sepharose coupled rCD44s, rCD44v6 and rCD44v7. Eluted proteins were analyzed by MALDI-TOF. Suppl. Table 3 lists the proteins, where specific hits were obtained and indicates the number of specific hits. The vast majority of cellular CD44 ligands / associated molecules bound to rCD44s, rCD44v6 and rCD44v7 (Suppl. Figure 3B), which suggests preferential binding to / association with epitopes of CD44s. Few molecules selectively bound rCD44v6 and/or rCD44v7. In HSPC lysates this has been complement component C3 (rCD44v6), radixin and superoxide dismutase (rCD44v6 and rCD44v7) (Suppl. Table 3). Sorting the recombinant CD44 binding/associated molecules according to protein classes revealed that a considerable number of cellular proteins from HSPC and BM-StrC were adhesion molecules, members of the cytoskeleton, proteases or metabolic enzymes. Whereas in BM-StrC rCD44 preferentially binds adhesion and cytoskeletal molecules, binding of metabolic enzymes was dominating in HSPC and covered close to 50% of the eluted proteins. (Suppl. Figure 3C).

In addition to matrix protein- and cytokine-induced instructions, the crosstalk with niche cells is of major importance in the regulation of HSPC. Thus, having demonstrated binding of cellular proteins to recombinant CD44, we searched, whether CD44v6 and CD44v7 contribute to the binding of HSPC to BM-StrC. CFSE-labeled HSPC were seeded on a monolayer of BM-StrC. After 4h incubation, non-adherent HSPC were washed off, adherent cells were lysed and CFSE recovery was evaluated in a fluorescence ELISA reader. Adhesion of CD44v6/v7^{ko} HSPC to wt, CD44v7^{ko} and CD44v6/v7^{ko} BM-StrC was reduced compared to wt HSPC adhesion (Figure 2G). Inhibition of binding to wt BM-StrC after preincubation of wt, CD44v7^{ko} and CD44v6/v7^{ko} HSPC with anti-panCD44, anti-CD44v6 and anti-CD44v7 confirmed the contribution of HSPC CD44v6 to BM-StrC binding (Figure 2H). Importantly, HSPC binding to CD44v7^{ko} and CD44v6/v7^{ko} BM-StrC was strongly reduced such that CD44v6/v7^{ko} HSPC hardly bound to CD44v6/v7^{ko} BM-StrC and anti-panCD44 exerted no additional inhibitory effect (Figure 2G and 2H).

In brief, HSPC CD44v6 and CD44v7 exert a minor contribution to matrix protein adhesion. Likely via associating molecules, HSPC CD44v6/v7 are engaged in SDF1, OPN and SCF binding. CD44v6 on HSPC supports adhesion to the BM-StrC. However, while HSPC CD44v6 provides a minor contribution, BM-StrC CD44v7 is essential for HSPC binding.

CD44 binding being known to initiate signals that promote motility, proliferation and apoptosis resistance, we proceeded to control for the impact of HSPC CD44v6 and CD44v7. Notably, proliferation and apoptosis resistance of activated leukocyte and solid tumor cells frequently is associated with CD44v6 expression [6].

CD44 and HSPC migration

The engagement of HSPC CD44 in HA, FN and chemokine binding [43,44] suggested a contribution to HSPC homing and migration.

As revealed by transwell migration, CD44^{wt}, CD44v7^{ko} and CD44v6/v7^{ko} HSPC readily migrated towards the multifactorial stimulus 20%FCS as well as towards HA, FN and less pronounced coll IV. Migration of CD44v7^{ko} HSPC towards FN and of CD44v6/v7^{ko} HSPC towards HA and FN was significantly reduced compared to wt HSPC migration (Figure 3A). Inhibition of HSPC transwell migration after preincubation with anti-panCD44, anti-CD44v6 and anti-CD44v7 confirmed the contribution of panCD44 and CD44v6 to migration towards HA and FN, and of CD44v7 to migration towards FN (Figure 3B). Video microscopy, where migration of individual HSPC was evaluated over a period of 16h confirmed reduced migratory activity of CD44v6/v7^{ko} HSPC on HA-coated plates as well as inhibition of wt and CD44v6/v7^{ko} HSPC migration in the presence of anti-panCD44 and of wt HSPC in the presence of anti-CD44v6 (Figure 3C).

HSPC also migrated towards IL6, SDF1, OPN, SCF, GMCSF and bFGF. HSPC CD44v6/CD44v7 contributed to migration towards OPN and SDF1. Distinct to adhesion, CD44v6/v7^{ko} HSPC migration towards IL6 was reduced. Migration of HSPC towards SCF, GMCSF and bFGF appeared CD44v6- and CD44v7-independent as no change in migratory activity of wt versus CD44v7^{ko} and CD44v6/v7^{ko} HSPC was observed (Figure 3D). To further control for the impact of CD44s, CD44v6 and CD44v7 on migration towards these cytokines, wt HSPC were preincubated with anti-panCD44, anti-CD44v6 or anti-CD44v7. Anti-CD44 did not interfere with migration towards GMCSF, but inhibited migration towards IL6, SDF1, OPN, SCF and, weakly, bFGF, which confirms a contribution of HSPC CD44 to migration. Migration towards IL6 was also inhibited in the presence of anti-CD44v6 and migration towards OPN and SDF1 was inhibited in the presence of anti-CD44v6 or anti-CD44v7 (Figure 3E).

The migration of individual CFSE-labeled HSPC on BM-StrC was evaluated by video microscopy. Migration of wt and CD44v6/v7^{ko} HSPC on wt BM-StrC did not significantly differ, whereas migration on CD44v6/v7^{ko} BM-StrC was significantly reduced. Migration on wt BM-StrC was strongly reduced in the presence of anti-panCD44, wt HSPC migration on wt BM-StrC was also reduced in the presence of

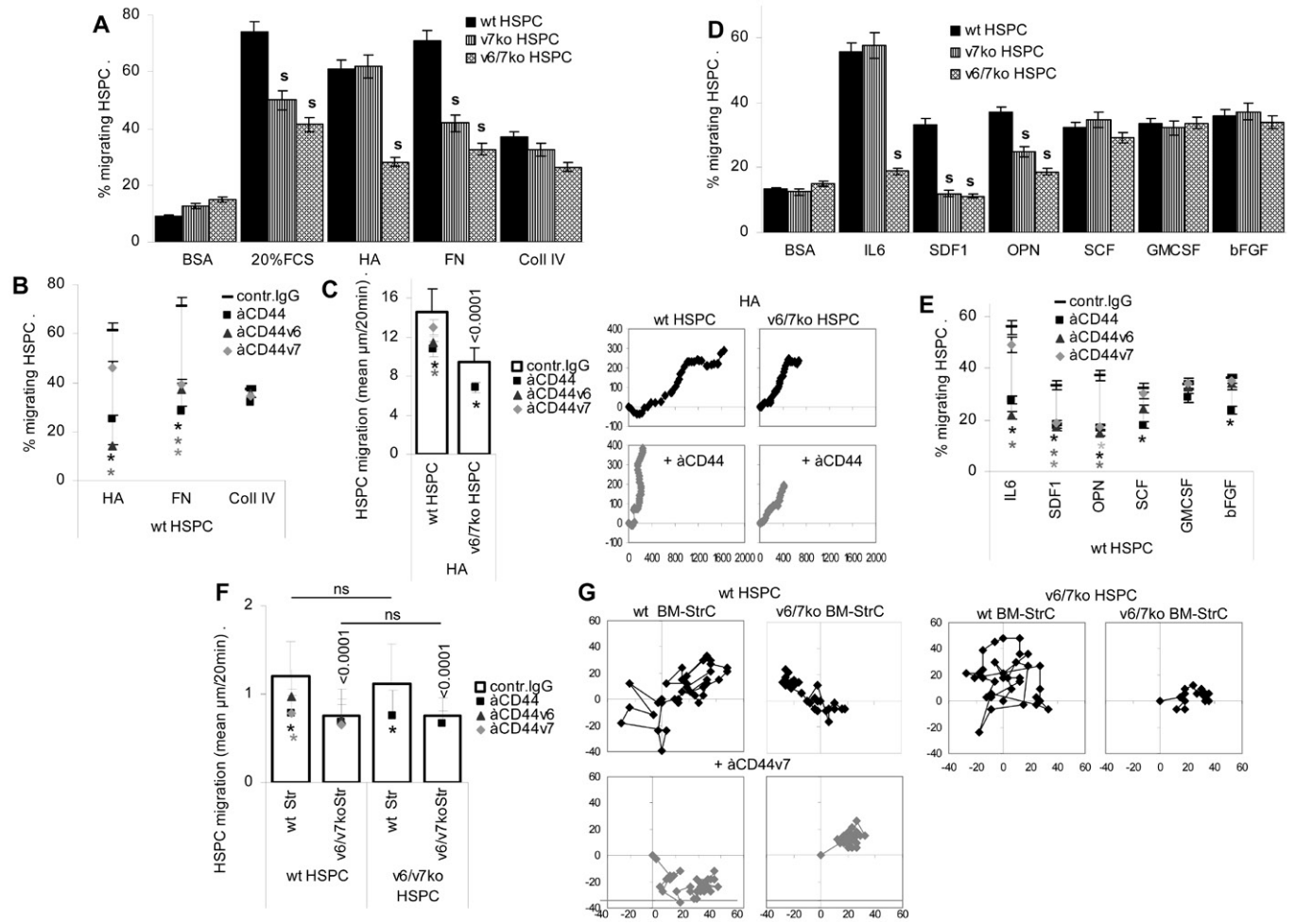

Figure 3: The impact of HSPC and BM-StrC CD44 on cell migration: (A,D) wt, CD44v7^{ko} and CD44v6/v7^{ko} HSPC were seeded in the upper part of a Boyden chamber, the lower part contained (A) BSA (control), 20% FCS, HA, FN or Coll IV or (D) BSA, IL6, SDF1, OPN, SCF, GMCSF or bFGF as stimulus. Cells in the lower chamber were counted after 6 h incubation. The mean percent ± SD (triplicates) of migrating HSPC is shown; significant differences between wt, CD44v7^{ko} and CD44v6/v7^{ko} HSPC: **s**. (B,E) Migration of HSPC towards matrix proteins and cytokines/chemokines in the presence of anti-CD44; the mean percent ± SD (triplicates) of migrating cells is shown; significant migration inhibition by anti-CD44: *. (C,F,G) CFSE-labeled wt and CD44v6/v7^{ko} HSPC were seeded on HA or a monolayer of wt or CD44v6/v7^{ko} BM-StrC in absence or presence of anti-CD44; migration of individual cells was followed for 16 h. The mean migration of 20 individual cells / 20 min and examples are shown; (C,F) p-values comparing wt and CD44v6/v7^{ko} HSPC migration on HA, wt and CD44v6/v7^{ko} BM-StrC as well as comparing migration on wt versus CD44v6/v7^{ko} BM-StrC are indicated, significant inhibition by anti-CD44: *.
HSPC migration is significantly influenced by HSPC CD44v6 and BM-StrC CD44v7.

anti-CD44v7 (Figure 3F and 3G). These findings indicated that BM-StrC CD44v7 plays an important role in HSPC migration on "niche" cells, but HSPC CD44v6 and CD44v7 apparently do not significantly contribute.

Taken together, distinct to adhesion, HSPC motility towards matrix proteins, cytokines and chemokines is significantly affected by HSPC CD44v6 and/or CD44v6-associated molecules. Neither HSPC CD44v6 nor CD44v7 are engaged in HSPC moving on BM-StrC. Instead, BM-StrC CD44v7 promotes motility of HSPC. The ligand for BM-StrC CD44v7 on HSPC remains to be defined.

HSPC CD44 and maintenance of quiescence

The interaction of HSPC with the niche is important for maintaining quiescence and preventing undue maturation [45-47]. Both HSPC CD44-HA and -BM-StrC binding could initiate signals that promote quiescence or interfere with undue division. A potential contribution of HSPC CD44v6 and CD44v7 has not yet been explored.

We first controlled for the contribution of CD44 to BMC and HSPC proliferation. CFSE-labeled cells were seeded on BSA, HA or BM-StrC and cell division was evaluated after 24h-72h by CFSE dilution. HSPC divided significantly less frequently than bulk BMC. CD44v6/CD44v7 had no significant impact on BMC proliferation, but CD44v6/v7^{ko} HSPC divided more frequently than wt HSPC. HSPC quiescence was supported by HA and wt, but hardly by CD44v6/v7^{ko} BM-StrC. This accounted for wt and, less pronounced, CD44v6/v7^{ko} HSPC (Figure 4A, 4B, 4C). Finally, an antibody blockade of CD44 promoted wt HSPC proliferation, the effect being most pronounced after 72h culture on HA-coated plates. Independent of the stimulus, anti-panCD44 exerted no significant effect on the higher proliferation rate of CD44v6/v7^{ko} HSPC (Figure 4D). Thus, HSPC binding to HA or wt BM-StrC promotes maintenance of quiescence, which is supported by HSPC CD44v6/CD44v7.

CD44v6/v7 additionally affected cell cycle progression as evaluated by PI staining. When wt HSPC were cultured on HA or wt, but not

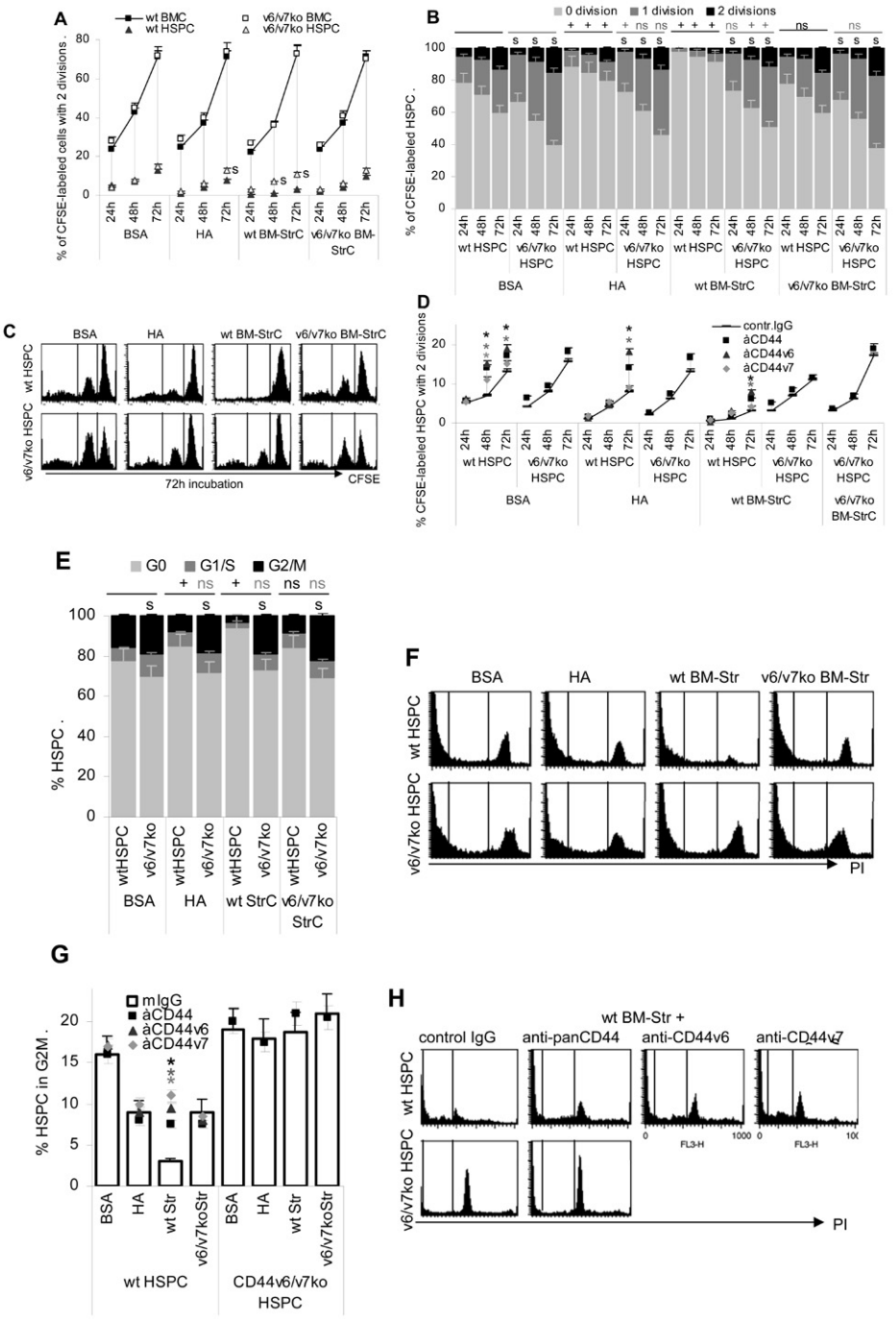

Figure 4: HSPC - BM-StrC binding supports HSPC quiescence and retards cell cycle progression: (A-D) BMC / HSPC were CFSE labeled. Cells were incubated for 24 h-72 h. Cell division was evaluated by CFSE-dilution (flow cytometry). (A) CFSE-labeled wt and CD44v6/v7^{ko} BMC and HSPC were seeded on BSA, HA or BM-StrC coated plates. Proliferation was evaluated after 24 h, 48 h and 72 h; mean percent ± SD (triplicates) of cells that had divided 2 times; significant differences between wt and CD44v6/v7^{ko} HSPC; (B,C) CFSE-labeled wt and CD44v6/v7^{ko} HSPC were seeded on BSA, HA, wt or CD446/v7^{ko} BM-StrC. The mean percent ± SD (triplicates) of HSPC that had not divided or divided 1-time or 2-times after 24 h-72 h of culture and a representative example after 72 h of culture are shown; significant differences between wt versus CD44v6/v7^{ko} HSPC: s; significant differences depending on the culture condition (BSA versus HA, wt or CD44v6/v7^{ko} BM-StrC): +; (D) the experiment described in (B) was repeated in the presence of anti-panCD44, -CD44v6 or -CD44v; the mean percent±SD (triplicates) of HSPC that had divided 2-times after 24h-72h of culture is shown; significant differences in the presence of anti-CD44: *. (E-H) Cell cycle progression (PI staining) was evaluated in CD44^{wt} and CD44v6/v7^{ko} HSPC seeded on BSA, HA, CD44^{wt} and CD44v6/v7^{ko} BM-StrC; (E,F) the mean percent ± SD (triplicates) of HSPC in G0, G1/S and G2/M and a representative example are shown; significant differences between CD44^{wt} and CD44v6/v7^{ko} HSPC: s; significant differences by culture condition: +; (G;H) the experiment shown in (E) was repeated in the presence of anti-panCD44, -CD44v6 or -CD44v7; the mean percent ± SD (triplicates) of HSPC in G0, G1/S and G2/M and a representative example are shown; significant differences in the presence of anti-CD44: *.

Wt HSPC divide less frequently than CD44v6/v7^{ko} HSPC. HA and wt BM-StrC promote HSPC quiescence; anti-panCD44 and -CD44v6 drive HSPC into proliferation. CD44v6/v7^{ko} HSPC are not efficiently protected from proliferation by HA or wt BM-StrC. Furthermore, CD44^{wt}, but not CD44v6/v7^{ko} HSPC transition from G1/S to G2/M is retarded by HA and wt BM-StrC. The wt BM-StrC-promoted retardation in cell cycle progression is strongly affected by anti-CD44.

CD44v6/v7^{ko} BM-StrC, a reduction was seen in the percent of cells in G1/S and G2/M. Furthermore, a slightly higher percent of CD44v6/v7^{ko} than wt HSPC were in G2/M (Figure 4E,4F). Anti-panCD44, -CD44v6 and -CD44v7 only affected wt HSPC cycling cultured on wt BM-StrC (Figure 4G,4H). We conclude that HSPC CD44v7 and CD44v6 contribute in maintaining HSPC in G0, particularly when supported by wt BM-StrC.

In advance of evaluating the impact of HSPC CD44v6 and CD44v7 on expression of central components regulating HSPC cycling, we controlled expression of relevant ligands in wt versus CD44v6/v7^{ko} BM-StrC. We did not see significant differences in SDF1, IL6, Wnt, BMP4, LIF, inhibin, CD105 and OPN expression, but TGFβ was expressed at a higher percentage of CD44v6/v7^{ko} than wt BM-StrC (Suppl. Figure 4A,4B). However, TGFβ upregulation on CD44v6/v7^{ko} BM-StrC was poorly reflected in TGFβ-regulated gene expression in HSPC. Irrespective of the culture condition, no differences were seen in Flt3 expression; CD126 expression was increased only in wt HSPC cultured on BM-StrC. CD117 expression was decreased in CD44v6/v7^{ko} HSPC cultured on CD44v6/v7^{ko} BM-StrC. In concern about Wnt-regulated genes, no differences were seen between wt and CD44v6/v7^{ko} HSPC in LEF1 and c-myc expression, which was increased upon culture on CD44v6/v7^{ko} BM-StrC. However, β-catenin and cyclinD1 expression was higher in CD44v6/v7^{ko} than wt HSPC, when cultured on BSA- or HA-coated plates. This difference was waved, when wt HSPC were cultured on wt or CD44v6/v7^{ko} BM-StrC, which promoted β-catenin and cyclinD1 upregulation (Figure 5A, 5B).

Taken together, in the absence of CD44v6/v7 on HSPC the crosstalk with HA and the stroma is impaired such that HSPC more rapidly divide, which is in line with the poorer recovery of HSPC from CD44v6/v7^{ko} mice (data not shown), a reduction in CD117^{+} cells, when cultured on CD44v6/v7^{ko} BM-StrC, the increased β-catenin and cyclinD1 expression in CD44v6/v7^{ko} HSPC and the failure to respond in cocultures with CD126 upregulation.

HSPC CD44 and apoptosis resistance

HSPC are characterized by relative apoptosis resistance [48,49]. A possible contribution of HSPC and/or BM-StrC CD44v6 and CD44v7 was not yet explored.

Apoptosis was evaluated by AnnV/PI staining. Culturing bulk BMC and HSPC on BSA- or HA-coated plates or on a monolayer of wt BM-StrC confirmed a lower apoptosis rate of HSPC than BMC. The difference between bulk BMC and HSPC became more pronounced, when cultured on wt BM-StrC as well as in the presence of 5µg/ml cisplatin, which sufficed to significantly increase apoptosis in bulk BMC, but hardly affected HSPC attached to HA or BM-StrC (Figure 6A and 6B). The higher apoptosis resistance of HSPC was confirmed by triple staining unseparated BMC and HSPC with AnnV, PI and anti-CD117, where the small population of CD117^{+} BMC also displayed high apoptosis resistance (Figure 6C).

The high apoptosis resistance of HSPC could be promising for attacking LIC. Notably, in activated leukocytes and epithelial tumors, the engagement of CD44 in apoptosis resistance mostly relies on CD44v6 [50-53]. Thus, we asked, whether low level CD44v6/CD44v7 expression in HSPC contributes to apoptosis protection. When CD44v7^{ko} and CD44v6/v7^{ko} HSPC were cultured in the presence of cisplatin on BSA- or HA-coated plates or on a monolayer of wt, CD44v7^{ko} or CD44v6/v7^{ko} BM-StrC, a significant increase in apoptotic CD44v6/v7^{ko} compared to wt HSPC was seen, whereas apoptosis resistance of CD44v7^{ko} HSPC

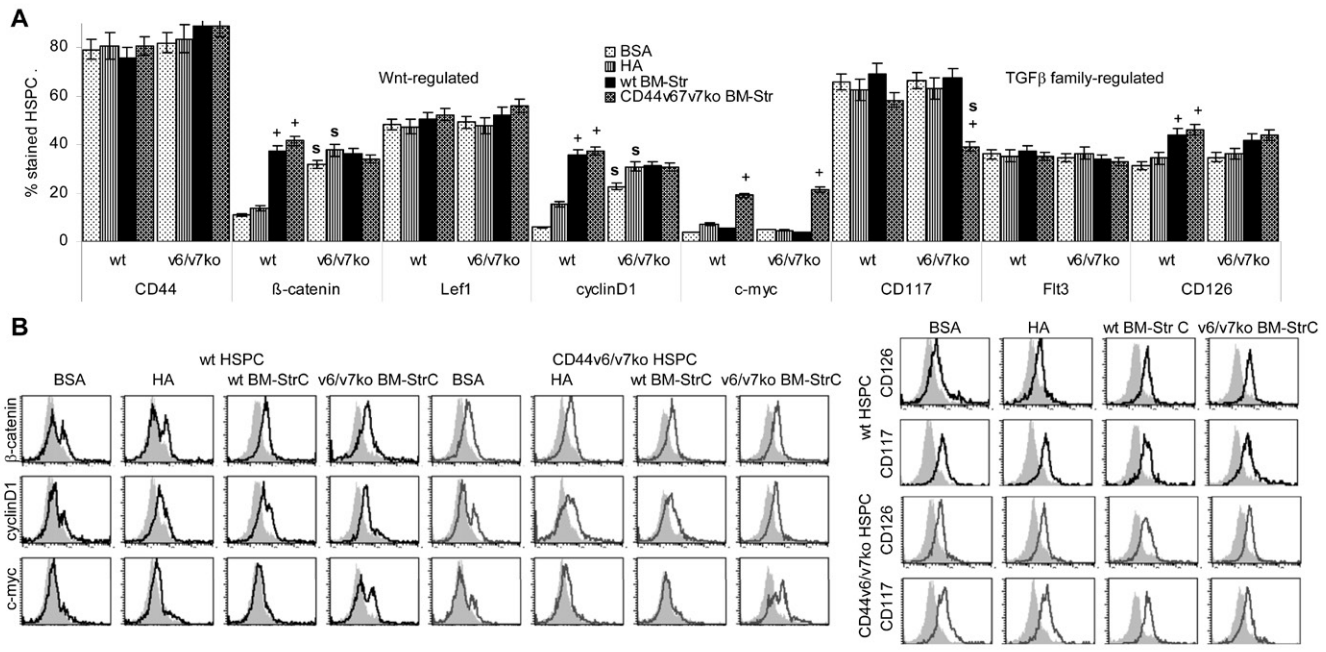

Figure 5: The impact of CD44v6 and CD44v7 on cell cycle progression: (A,B) Flow-cytometry analysis of expression of Wnt- and TGFβ-regulated genes in CD44^{wt} and CD44v6/v7^{ko} HSPC cultured on BSA, HA, wt or CD44v6/v7^{ko} BM-StrC; percent stained HSPC (mean ± SD, triplicates) and examples; significant differences by culture condition: +, significant differences between CD44^{wt} and CD44v6/v7^{ko} HSPC: **s**.
TGFβ upregulation in CD44v6/v7^{ko} BM-StrC (Suppl.Fig.4) was not accompanied by altered expression of TGFβ-regulated genes with the exception of a slight reduction in CD117^{+} cells. In addition, Wnt-regulated gene expression varied, which points towards a multifactorial process and significant contributions of CD44-independent components.

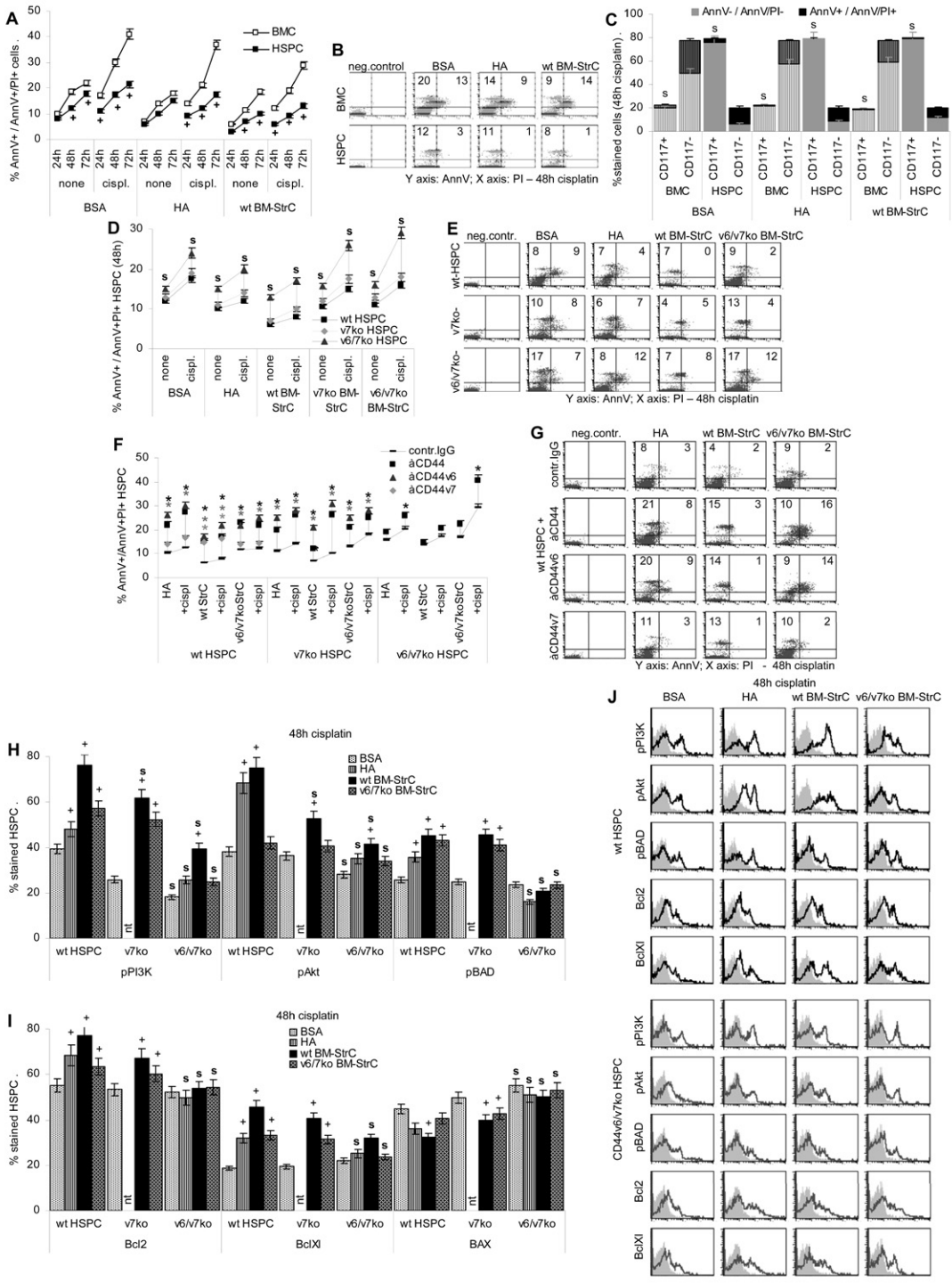

Figure 6: Apoptosis resistance of HSPC is supported by HA and BM-StrC: (A-C) Wt BMC and HSPC and (D-G) wt, CD44v7^{ko} and CD44v6/7^{ko} HSPC were cultured for 24h-72h in the absence or presence of 5µg/ml cisplatin on BSA, HA or BM-StrC. Where indicated cultures contained anti-CD44; apoptosis was evaluated by AnnV/PI staining; (A,B) Comparison of the % AnnV$^+$/AnnV$^+$/PI$^+$ cells in BMC versus HSPC; the mean percentage (±SD) (triplicates) of AnnV$^+$/AnnV$^+$/PI$^+$ cells and representative examples are shown; significant differences between BMC and HSPC: +; (C) repetition of the experiment shown in (A) and triple staining for CD117, AnnV and PI; the mean percentage (±SD) (triplicates) of CD117$^+$ and CD117$^-$ cells that are AnnV$^+$/PI$^+$ or AnnV$^-$/PI$^-$ are shown; significant differences in AnnV$^+$/PI$^+$ CD117$^+$ versus CD117$^-$ cells: s; (D,E) wt, CD44v7^{ko} and CD44v6/v7^{ko} HSPC were cultured in the presence or absence of cisplatin on BSA, HA or BM-StrC coated plates; the mean percentage (±SD) (triplicates) of AnnV$^+$/AnnV$^+$/PI$^+$ cells and representative examples are shown; significant differences between wt, CD44v7^{ko} and CD44v6/v7^{ko} HSPC: **s**; (F,G) wt, CD44v7^{ko} and CD44v6/v7^{ko} HSPC were cultured in the presence or absence of cisplatin on BSA, HA or BM-StrC coated plates; the cultures contained control IgG or anti-panCD44, -CD44v6 or -CD44v7; the mean percentage (±SD) (triplicates) of AnnV$^+$/AnnV$^+$/PI$^+$ cells and representative examples are shown; significant differences in the presence of anti-CD44: *; (H-J) Flow-cytometry analysis of anti-apoptotic molecules in wt, CD44v6/v7^{ko} and CD44v7^{ko} HSPC cultured for 48 h on BSA, HA, CD44^{wt} or CD44v6/v7^{ko} BM-StrC in the presence of cisplatin; mean percent ± SD (triplicates) of stained HSPC and representative examples; significant differences by culture condition: +; significant differences between wt, CD44v6/v7^{ko} and CD44v7^{ko} HSPC: **s**.

was not affected. The loss in apoptosis resistance of CD44v6/v7^{ko} HSPC was most pronounced in cocultures with CD44v6/v7^{ko} or CD44v7^{ko} BM-StrC, pointing towards wt BM-StrC providing an anti-apoptotic trigger (Figure 6D, 6E). Nonetheless, it should be stressed that even CD44v6/v7^{ko} HSPC cells displayed higher apoptosis resistance than unseparated BMC.

The impact of HSPC CD44v6 on apoptosis resistance was confirmed by antibody blocking. Apoptosis of wt HSPC cultured on HA was significantly increased in the presence of anti-panCD44 or anti-CD44v6, but less efficiently in the presence of anti-CD44v7. The impact of anti-CD44 also was strong on CD44v7^{ko}, but weak on CD44v6/v7^{ko} HSPC. Similar results were obtained, when culturing wt, CD44v7^{ko} and CD44v6/v7^{ko} HSPC on wt BM-StrC. To differentiate between the impact of CD44v6 and CD44v7 on HSPC versus BM-StrC, the antibody blocking experiment was repeated with wt HSPC cocultured with CD44v6/v7^{ko} BM-StrC. Anti-CD44v6, but not anti-CD44v7 promoted apoptosis (Figure 6F and 6G). Thus, HSPC CD44v6 contributes to apoptosis resistance, which is supported by HA and BM-StrC.

Searching for HSPC CD44v6-initiated signals that might account for increased apoptosis resistance provided no evidence for an engagement of death receptors. CD95L was the only death receptor consistently upregulated in cisplatin-treated CD44v6/v7^{ko} HSPC. Furthermore, a slight downregulation of CD95 and a minor upregulation of TRAIL and TNFR1 in cocultures with BM-StrC were independent of HSPC CD44v6/v7 expression (Suppl. Figure 5A). The analysis of caspase activity confirmed independence of death receptors inasmuch as caspase8 activity was similar in CD44^{wt} and CD44v6/v7^{ko} HSPC with lower level expression in HA- or wt BM-StrC-supported HSPC. Instead, HA and wt BM-StrC more efficiently protected cisplatin-treated wt HSPC than CD44v6/v7^{ko} HSPC from caspase9 cleavage and caspase3 activation. CD44v6/v7^{ko} BM-StrC exerted a weaker effect than wt BM-StrC on caspase expression / activation (Suppl. Figure 5B and 5C).

Unimpaired death receptor expression, but reduced HSPC apoptosis pointed towards HA and BM-StrC supporting activation of the anti-apoptotic pathways. PI3K phosphorylation was significantly strengthened, when HSPC were protected by HA or BM-StrC. The same accounted for pBAD, Bcl2 and BclXl expression; upregulation of pAkt and reduced BAX expression was mostly seen on HA- or wt BM-StrC-coated plates. These findings differed significantly from those of cisplatin-treated CD44v6/v7^{ko} HSPC, where pPI3K, pAkt and pBAD expression was hardly promoted by HA or BM-StrC and BAX expression was higher. Distinct to CD44v6/v7^{ko} HSPC, the response of CD44v7^{ko} HSPC did not significantly differ from that of wt. HSPC (Figure 6H-6J).

Thus, HSPC CD44v6 is important for initiating anti-apoptotic signaling via HA or BM-StrC binding. Taken together, HSPC highly express CD44 and weakly CD44v6/v7. Mostly CD44s accounts for matrix binding. Upon engagement with HA or BM-StrC, CD44v6 acts as signaling initiator in HSPC, which supports motility, quiescence and apoptosis resistance.

Discussion

There is ample evidence for the engagement of CD44 in the crosstalk between HSPC and LIC with the osteogenic niche [54]. Accordingly, anti-CD44 has been used for HSPC mobilization [55] and as a therapeutics in AML and CML [36,37]. On the other hand, due to abundant CD44 expression, a blockade of CD44, particularly under stress and poor health condition, can have deleterious consequences [39,56]. Distinct to CD44s, CD44 variant isoforms are not or weakly expressed on hematopoietic cells [57], but CD44v expression frequently is upregulated in leukemia [58]. Thus, we hypothesized that severe side effects possible can be avoided by the therapeutic use of CD44v-specific antibodies, which we explored for CD44v6 and CD44v7. Using HSPC and, as a surrogate of the osteogenic niche, BM-StrC of CD44^{wt}, CD44v7^{ko} and CD44v6/v7^{ko} mice as well as CD44v6- and CD44v7-specific antibodies, we asked whether and which HSPC activities are supported by CD44v6/CD44v7. The answers to this question should provide hints towards a focused attack of LIC, where we particularly discuss the contribution of CD44v6 and CD44v7 to HSPC niche embedding, homing, quiescence and apoptosis resistance under the angel of the possible impact on LIC. A simplified overview is given in Supplementary Figure 6.

CD44v6 and CD44v7 expression in HSPC and impact on adhesion to the BM-Str

Leukemia cells do not essentially express CD44v isoforms. However, if expressed, expression levels are high in most instances. However, distinct to CD44s expression, expression of CD44v6 and CD44v7 is low in HSPC. Thus, it can be expected that a therapeutic CD44v6-specific antibody will preferentially bind to the leukemia cell and may not significantly attack HSPC.

Adhesion of HSPC to HA, FN and coll IV mostly involves CD44s, although CD44v6 contributes to HA adhesion. An apparent contribution of CD44v6 to FN and coll IV adhesion possibly is due to CD44-associated integrins. These findings argue against a selective, CD44s-independent contribution of CD44v to matrix adhesion, but suggest that the CD44 binding affinity is modulated by the activation state of the cell, which can involve the insertion of variant exons [59]. This could be a (minor) advantage for attacking CD44v6^{+} LIC by anti-CD44v6.

Distinct to matrix adhesion, CD44 isoforms differently contribute to cytokine and chemokine adhesion. There is no evidence for a contribution of HSPC CD44 to IL3 and GM-CSF binding. However, HSPC CD44 contributes to SCF, SDF1, OPN and IL6 binding. Except for IL6, antibody blocking indicating an engagement of CD44v6 and CD44v7. Notably, cytokine / chemokine binding is not waved in the absence of CD44v6 and/or CD44v7 and anti-panCD44 reduces, but does not abolish binding, which indicates a contribution of additional HSPC membrane molecules in cytokine / chemokine binding. Though the impact of a CD44v6 blockade on cytokine-promoted recruitment and retention of LIC in the niche remains to be explored, the importance of the SDF1-CXCR4 interaction in HSPC retention in the niche is well established [60,61]. As SDF1 binding to HSPC CXCR4 was efficiently inhibited by anti-CD44v6, it can be expected that anti-CD44v6 severely affects embedding of CD44v6 expressing LIC.

CD44v6/CD44v7 also contributes to the binding of HSPC to BM-StrC. Notably, binding is most strikingly reduced in the absence of CD44v6/v7 on both HSPC and BM-StrC, which was confirmed by antibody inhibition, where anti-panCD44 did not affect low binding of CD44v7^{ko} and CD44v6/v7^{ko} HSPC to CD44v6/v7^{ko} BM-StrC and poorly the binding of wt HSPC to CD44v6v7^{ko} BM-StrC. Thus, it is mostly the expression of CD44v7 / CD44v6/v7 on BM-StrC that supports HSPC adhesion.

In brief, HSPC CD44 significantly contributes to matrix protein, cytokine/chemokine and BM-StrC adhesion. A blockade of CD44v6 exerts a minor effect on HSPC binding, which can be expected to be stronger on CD44v6 expressing LIC.

CD44 and HSPC homing / migration

Leukemia therapy frequently relies on autologous HSPC transplantation [62]. Thus, it became important to know, whether LIC compete with HSC not only for the niche, but also for homing and whether CD44v6/CD44v7 contribute to migration and niche homing.

The analysis of HSPC migration confirmed the studies on the contribution of CD44 to HSPC adhesion. Yet, the impact of HSPC CD44v6 to migration was stronger than to adhesion. This accounted for migration towards matrix proteins as well as towards chemokines / cytokines. In concern about migration towards matrix proteins the strong impact of CD44v6 and CD44v7 on migration towards FN should be mention, CD44 being described as a FN ligand [13,63]. Notably, too, migration towards IL6 was strikingly impaired in the absence of CD44v6/v7 and was most strongly inhibited by anti-CD44v6. Migration of CD44v7^{ko} and CD44v6/v7^{ko} HSPC towards SDF1 was reduced to background levels and was strongly inhibited by anti-panCD44, -CD44v6 and -CD44v7, indicating major importance of these two splice variants in following a SDF1 gradient. Furthermore, OPN binding and migration towards OPN was impaired in CD44v6/v7^{ko} HSPC, which fits the selective CD44v6/v7 binding of OPN and pronounced migration and invasion triggered by OPN binding [64-67]. Finally, migration of HSPC on BM-StrC confirmed *in vivo* findings [68] on a strong contribution of BM-StrC CD44v7.

These findings suggest that anti-CD44v6 may hamper transplanted HSPC homing, even if LIC express CD44v6 at a higher level. On the other hand, there is evidence that LIC may rely to a greater extent on selectins and their ligands for homing and engraftment than HSC [69]. Thus, during autologous HSPC transplantation, at least, a concomitant blockade of CD44v6 and selectins or solely anti-selectins may be beneficial. In concern about the strong contribution of BM-StrC CD44v7 to HSPC attraction, we suggests that anti-CD44v7 may not be suited for leukemia therapy, as niche embedding of HSPC transplants will also become severely affected.

Defining the cellular ligands of HSPC (and LIC) could provide further hints towards a preferential / selective blockade of LIC versus HSPC. Though the cellular ligands of CD44v6 and CD44v7 are not comprehensively defined, pulldown studies point towards a dominance of glycoproteins and glycosylation modifying enzymes that cope with oxidative stress, where SOD, the first line of defense against oxidative stress [70], was only recovered in HSPC lysates and was captured particularly by CD44v6 and CD44v7. Another protein selectively trapped by CD44v6/v7 was C3. We have reported before that C3 expression is linked to CD44v6 expression [53,71]. The high recovery of C3 in rCD44v6 eluates was unexpected. However, as elegantly elaborated by Lee and Ratajczak [72], C3 can drive CXCR4 into lipid rafts, where it associates with CD44v6 thereby strengthening the CXCR4 – SDF1 axis that helps retaining HSPC/LIC in the niche. In concern about leukemia therapy, this might imply that anti-CD44v6 treatment also interferes with the leukemia growth-promoting activity of the innate immune system.

CD44 and HSPC quiescence and apoptosis resistance

HSPC divide lifelong, but rarely [73], which was confirmed comparing the division rate of HSPC with that of unseparated BMC. The HSPC division rate became further decreased when seeded on HA and most pronounced when in contact with BM-StrC. BM-StrC are known to support HSPC dormancy versus activation [74] and a contribution of CD44 is discussed [75,76]. We here demonstrated that HSPC CD44v6 is engaged in HSPC quiescence as proliferation is upregulated in the presence of anti-CD44v6 as well as in CD44v6/v7^{ko} HSPC. Furthermore, anti-panCD44 does not affect CD44v6/v7^{ko} HSPC proliferation. Finally, there was evidence for a contribution of BM-StrC CD44v6/v7, wt HSPC dormancy being not supported by CD44v6/v7^{ko} BM-StrC.

HSPC CD44v6/v7 also influences cell cycle progression [77]. Fewer CD44^{wt} than CD44v6/v7^{ko} HSPC were in G2/M when cultured on HA or CD44^{wt}, but not CD44v6/v7^{ko} BM-StrC and anti-panCD44, -CD44v6 and -CD44v7 drive wt HSPC into mitosis when cultured on wt BM-StrC; anti-panCD44 had no effect on the percentage of CD44v6/v7^{ko} HSPC in G2/M. Thus, HSPC and BM-StrC CD44v6/v7 contributed to HSPC quiescence.

The strong contribution of CD44v6/v7-competent BM-StrC to HSPC quiescence prompted us to search for quiescence regulating protein expression in wt versus CD44v6/v7^{ko} BM-StrC, where we only noted upregulation of TGFβ in CD44v6/v7^{ko} BM-StrC. This finding was opposing our expectation, as TGFβ is a potent inhibitor of HSPC proliferation [78]. Nonetheless, we proceeded to control for changes in the most prominent TGFβ regulated genes in CD44v6/v7-competent versus deficient HSPC [79]. However, no differences were observed with the exception of CD117 downregulation in CD44v6/v7^{ko} HSPC cultured on CD44v6/v7^{ko} BM-StrC. Further experiments are needed to clarify the impact of TGFβ that may have been blurred by the redundancy of TGFβ signaling in hematopoiesis [80].

Wnt signaling has emerged as another important factor in HSC quiescence [81]. From the prominent Wnt targets, β-catenin and cyclinD1 expression were higher in CD44v6/v7^{ko} HSPC and c-myc became upregulated in cocultures with CD44v6/v7^{ko} BM-StrC. Instead, in wt HSPC β-catenin and cyclinD1 became upregulated in BM-StrC cocultures. These findings point towards a minor impact of BM-StrC, particularly BM-StrC CD44v6/v7 on Wnt signaling in HSPC. However, Wnt effects being highly context and dose dependent [82-84], it is difficult to precisely define its role in HSPC maintenance. Nonetheless, the association of CD44 with Wnt signaling is amply demonstrated for HSPC [85] and LIC [86], where our findings suggest that (i) HA and BM-StrC – apparently CD44v6/v7-independent - support HSPC quiescence and (ii) HSPC CD44v6/v7 supports the transport the respective signals.

In concern about attacking LIC via anti-CD44v6, we want to stress the point that regulation of HSPC and LIC quiescence differ fundamentally, only HSPC quiescence is linked to the hypoxic state in the niche [87,88]. Besides maintaining redox homeostasis by low oxygen production due to the minimal metabolic rate [89,90], HSPC generate energy mainly via anaerobic metabolism maintaining a high rate of glycolysis [91,92] by transcriptional activation of genes encoding glucose transporters, glycolytic enzymes and metabolic regulatory enzymes [93]. Notably, the intracellular domain of CD44 acts as co-transcription factor, which promotes expression of several genes directly associated with aerobic glycolysis [94,95]. Though neither the TGFβ nor the Wnt pathway are strongly affected in CD44v6/v7^{ko} HSPC, anti-CD44v6 could well provide a means to distort LIC quiescence, which is distinctly regulated [96], e.g. the CD44v6 ligand OPN negatively regulates the number of HSC in the BM niche [97], but promotes CIC expansion via activation of the PI3K/Akt pathway [98].

The distinction between HSPC and LIC also accounts for apoptosis resistance, which in HSPC is dictated by low oxygen production [88] and only partly by activation of anti-apoptotic signaling cascades, whereas the latter dominates in LIC and frequently is initiated via CD44v6-associated receptor tyrosine kinases [99].

Cisplatin treatment revealed reduced apoptosis resistance of CD44v6/v7^{ko} compared to wt HSPC. The difference to wt HSPC became most pronounced, when HSPC were cultured on HA-coated plates or were cocultured with wt BM-StrC, which both exerted a strong apoptosis-protective effect on wt HSPC. A selective contribution of BM-StrC CD44v6/v7 appears likely, as wt BM-StrC exerted a stronger protective effect than CD44v6/v7^{ko} BM-StrC. Yet, HSPC CD44v6 is of major importance, as apoptosis resistance was blocked by anti-CD44v6 in wt HSPC, but not by anti-panCD44 in CD44v6/v7^{ko} HSPC. Apoptosis protection relied on activation of the PI3K/Akt apoptosis protective pathway, which was stimulated by HA and BM-StrC. Thus, HSPC CD44v6 crosslinking promotes cytotoxic stress protection via activation of PI3K. Multiple pathways of downstream signal transduction via PTEN, MAPKs or NF□B liberation are discussed [100]. We previously demonstrated that CD44v6-promoted drug resistance proceeds mostly via activation of the MAPK pathway [53]. Another, well known pathway of apoptosis resistance proceeds via CD44v6-associated MET, which is initiated by HGF binding to CD44v6 [101]. HA-stimulated CD44v6 in transformed cells also activates the insulin-like growth factor-1 receptor and PDGFR [102,103]. Thus there is abundant evidence, which suggests that anti-CD44v6 could efficiently drive LIC into apoptosis, while HSPC survival, where apoptosis resistance relies mostly on low oxygen production, may not become severely affected.

We are currently progressing to identify CD44v6-associating molecules on HSPC to further clarify the contribution of CD44v6-initiated signal transduction in HSPC quiescence. First to note, data so far (Suppl. Table 4) reveal a striking overlap with molecules binding rCD44 (Suppl. Table 3), which implies mostly lateral associations to be catched by the pulldown. In general, coimmunoprecipitation confirmed the association of CD44 with integrins and selectins [42,104] and points towards FcγRIII (CD16) also associating with CD44. Cytoskeletal linker proteins, repeatedly described to associate with CD44 [105,106], apparently more readily associate with CD44v6 than CD44s. As activation-initiated conformational changes support ERM protein phosphorylation [105], we interpret the finding in the sense that CD44v6 expression is linked to a state of HSPC activation initiated by HA or BM-StrC binding. Finally, several proteases and kinases preferentially associated with CD44v6. As discussed above, the high recovery of metabolic enzymes and stress response proteins including several HSP may well be important for HSPC survival in the hypoxic osteogenic niche [46,107], whereas the abundance of proteases, including cathepsin G could facilitate the timely release of HSPC from the niche [108,109]. CD44v6-associated molecules in LIC remain to be identified and are expected to provide hints for a potential LIC-selective CD44v6 complex to be attacked.

Conclusion and Outlook

Steady state hematopoiesis of CD44v7^{ko} and CD44v6/v7^{ko} mice appears normal [68]. Nonetheless, and even though CD44v6/CD44v7 expression is low, we could elaborate that HSPC CD44v6 and CD44v7 contribute to the crosstalk with HA and BM-StrC, where HSPC CD44v6 is particularly involved in migration and apoptosis resistance, with a minor contribution to cell cycle regulation and proliferation (differentiation). Low level expression and the dispensability of CD44v6/v7 for steady state hematopoiesis should facilitate a CD44v-specific therapy in leukemia that overexpress CD44 variant isoforms as repeatedly demonstrated for CD44v6 [110-114], CD44v9 [115] and CD44v10 [116]. To strengthen the therapeutic efficacy of a CD44v6-directed therapy and based on our results that CD44v6 contributes to motility and apoptosis resistance, we suggest bispecific antibodies that recognize with the second arm CD44v6-associated molecules expressed in LIC, but not or weakly in HSPC [56]. A possible candidate could be a bispecific anti-CD44v6/-ALDH antibody, ALDH associating with CD44 and being upregulated in LIC [117]. Of interest will also be a bispecific antibody targeting CD44v6 and SOD that appears to be selectively associated with CD44v6 [70].

Taken together, after a thorough evaluation of the impact of CD44v6/v7 on HSPC and BM-StrC, we conclude that attacking CD44v6 expressing LIC via anti-CD44v6 should be a save and promising approach.

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