Cooperativity of CD44 and CD49d in Leukemia Cell Homing, Migration, and Survival Offers a Means for Therapeutic Attack

Vibuthi Singh, Ulrike Erb and Margot Zöller

*J Immunol* published online 14 October 2013
http://www.jimmunol.org/content/early/2013/10/11/jimmunol.1301543

Supplementary Material  http://www.jimmunol.org/content/suppl/2013/10/15/jimmunol.1301543
3.DC1

Subscription  Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions  Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts  Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Cooperativity of CD44 and CD49d in Leukemia Cell Homing, Migration, and Survival Offers a Means for Therapeutic Attack

Vibuthi Singh,*† Ulrike Erb,*† and Margot Zöller*†

A CD44 blockade drives leukemic cells into differentiation and apoptosis by dislodging from the osteogenic niche. Because anti-CD49d also supports hematopoietic stem cell mobilization, we sought to determine the therapeutic efficacy of a joint CD49d/CD44 blockade. To unravel the underlying mechanism, the CD49d− EL4 lymphoma was transfected with CD49d or point-mutated CD49d, prohibiting phosphorylation and FAK binding; additionally, a CD44− Jurkat subline was transfected with murine CD44, CD44 with a point mutation in the ezrin binding site, or with cytoplasmic tail-truncated CD44. Parental and transfected EL4 and Jurkat cells were evaluated for adhesion, migration, and apoptosis susceptibility in vitro and in vivo. Ligand-binding and Ab-blocking studies revealed CD44-CD49d cooperation in vitro and in vivo in adhesion, migration, and apoptosis resistance. The cooperation depends on ligand-induced proximity such that both CD44 and CD49d get access to src, FAK, and paxillin and via lck to the MAPK pathway, with the latter also supporting antiapoptotic molecule liberation. Accordingly, synergisms were only seen in leukemia cells expressing wild-type CD44 and CD49d. Anti-CD44 together with anti-CD49d efficiently dislodged EL4-CD49d/Jurkat-CD44 in bone marrow and spleen. Dislodging was accompanied by increased apoptosis susceptibility that strengthened low-dose chemotherapy, the combined treatment most strongly interfering with metastatic settlement and being partly curative. Ab treatment also promoted NK and Ab-dependent cellular cytotoxicity activation, which affected leukemia cells independent of CD44/CD49d tail mutations. Thus, mostly owing to a blockade of joint signaling, anti-CD44 and anti-CD49d hamper leukemic cell settlement and break apoptosis resistance, which strongly supports low-dose chemotherapy. The Journal of Immunology, 2013, 191: 000–000.

The leukocyte homing receptor CD44 (1) is expressed on most leukocytes, with the intensity of expression varying during leukocyte development as well as during leukocyte activation, where hematopoietic stem cells (HSCs), activated leukocytes, and memory T cells express CD44 at a high level (2, 3). CD44 is required for HSC- and leukemia-initiating cell homing in the osteogenic niche (4), which guarantees survival and prevents differentiation (5–9). Besides CD44, CD49d is engaged in HSC homing (10–12), transendothelial cell migration (13, 14), and mobilization (15–17). Both molecules (CD44 and CD49d) also contribute to hematopoietic cell proliferation (18, 19) and apoptosis resistance (20, 21). Taking into account that in activated leukocytes CD44 and CD49d associate such that CD44 gains access to CD49d-associated signaling molecules and CD49d to those of CD44 (22, 23), we speculated that in leukemia cells, too, CD44 and CD49d may jointly promote leukemia survival.

Hyaluronic acid (HA) is the major CD44 ligand (24). CD44 also binds to the CD49d ligand fibronectin (FN) (25, 26). HA binding initiates or modulates most CD44 activities (27). CD44 ligand binding can induce upregulation of additional adhesion molecules that strengthen adhesion and migration (28, 29). CD44-supported hematopoietic and leukemia cell migration relies on binding-induced interactions of the CD44 cytoplasmic tail with the actin cytoskeleton through ankyrin and ezrin-radixin-moesin (ERM) proteins (30, 31), whereby CD44 is guided to the leading edge of migrating cells (32). Binding of ERM proteins to CD44 requires an exchange of Ser325 versus Ser291 phosphorylation (33), allowing activated ERM proteins to bind to CD44 as well as the actin cytoskeleton (34). An additional central event in CD44-mediated cytoskeletal reorganization is Rac1 activation via the guanine exchange factors Vav1 and Vav2 (30), with their phosphorylation being mediated by src family kinases (35). Because CD44 is constitutively associated with src (36), CD44 binding to HA can initiate cytoskeletal reorganization as well as downstream signals to promote proliferation via activation of the MAPK and the INK pathway (37–40). Finally, CD44 crosslinking via HA can protect from apoptosis via multiple pathways, with activation of anti-apoptotic proteins via the PI3K/Akt pathway being repeatedly reported for HSCs and leukemia (41–43).

Similar to CD44, CD49d is engaged in HSC homing, motility, and apoptosis protection (12, 20). Ligand binding is accompanied by paxillin binding, which promotes FAK activation and formation of a signaling complex that, besides others, contains talin, α-actinin, and filamin (29, 44). Binding of paxillin is dictated by Ser388 phosphorylation, where mutation to Asp disrupts the α3 integrin–paxillin association (45).

*Department of Tumor Cell Biology, University Hospital of Surgery, 69120 Heidelberg, Germany; and †German Cancer Research Center, 69120 Heidelberg, Germany

V.S. and U.E. contributed equally to this work.

Received for publication June 12, 2013. Accepted for publication September 11, 2013.

This work was supported by a grant from the German Cancer Research Foundation (to M.Z.) and by German Research Foundation Grant ZO 40/14-1 (to M.Z.).

Address correspondence and reprint requests to Prof. Margot Zöller, Department of Tumor Cell Biology, University Hospital of Surgery, Im Neuenheimer Feld 365, 69120 Heidelberg, Germany. E-mail address: margot.zoeller@uni-heidelberg.de

The online version of this article contains supplemental material.

Abbreviations used in this article: ADCC, Ab-dependent cellular cytotoxicity; BM, bone marrow; E, EL4; ERM, ezrin-radixin-moesin; FN, fibronectin; HA, hyaluronic acid; HSC, hematopoietic stem cell; J, Jurkat; LN, lymph node; LNC, lymph node cell; MΔb, macrophage; mCD44, mutated CD44; mCD49d, mutated CD49d; PI, procidium iodide; SC, spleen cell; tcCD44, truncated CD44; wt, wild-type.

Copyright © 2013 by The American Association of Immunologists, Inc. 0022-1767/13/S16.00
We previously described that in activated T cells, CD44 cooperates with CD49d (23). Through the association of CD44 with CD49d, CD44 gains access to FAK and integrins gain access to src kinases and ERM proteins, such that the integrin–paxillin association becomes weakened and the GEM-integrated CD44/ezrin/integrin/FAK complex moves toward the leading edge, promoting cell migration (23). This finding is in line with anti-CD44 as well as anti-CD49d dislodging leukemic stem cells from their niche (5, 6, 17). The mutual access to the associated partner’s signaling pathways also affects T cell proliferation and apoptosis resistance (23). Considering the possibility of a concomitant therapeutic blockade of CD44 and CD49d in leukemia, in this study we sought to explore the underlying mechanisms. To facilitate differentiating between CD44-, CD49d-, and CD44/CD49d complex–mediated effects, we chose CD44+CD49d− EL4 and CD44+CD49d− Jurkat cells, which were transfected with wild-type (wt) and tail-mutated CD49d and CD44, respectively. This allowed distinguishing between the impacts of joint binding versus signal transduction. In vitro cooperativity of CD44 with CD49d in leukemia pre-Adherent cells were stained with crystal violet and lysed. Staining intensity

Materials and Methods

Cell lines

The murine thymoma EL4 and a CD44-negative subline of the human T leukemia line Jurkat were maintained in RPMI 1640 supplemented with 10% FCS, antibiotics, and glutamine. EL4 cells were transfected with CD49d (E-CD49d) and mutated CD44 cDNA (E-mCD49d); CD44-deficient Jurkat cells were transfected with CD44 (J-CD44), tailless CD44 (J-trCD44), and CD44 with a point mutation at the ezrin binding site (J-mCD44). Sequences and primers are listed in Supplemental Table IA. Transfected cells were cloned by limiting dilution in RPMI 1640/10% FCS supplemented with 250 μg/ml G418. The NK-sensitive YAC lymphoma cells were cultured in RPMI 1640/10% FCS.

Flow cytometry

Cells (1–3 × 10^5) were stained according to routine procedures. For intracellular staining of cytokines and signal transduction molecules, cells were fixed and permeabilized in advance. Apoptosis was evaluated by annexin V-FTTC/propidium iodide (PI) staining. Cell cycle progression was evaluated by PI staining. Staining was evaluated using a FACS Calibur (BD Biosciences). Analysis was performed by the CellQuest program. Experiments were repeated at least three times.

Immunohistology and fluorescence microscopy

Snap-frozen sections (5 μm) were fixed, incubated with Abs, washed, and exposed to biotinylated secondary Abs and alkaline phosphatase–conjugated avidin–biotin solution. Sections were counterstained with H&E. Cells on glass slides were fixed, permeabilized, blocked, incubated with primary Ab and fluorochrome-conjugated secondary Ab, blocked, incubated with secondary dye-labeled primary Ab, and washed. Slides were mounted in Elvanol, Digitalized images were generated using a Carl Zeiss LSM710 confocal microscope and Carl Zeiss AxioView 4.6 software.

Adhesion

Cells were seeded on matrix protein–coated 96-well plates. After washing, adherent cells were stained with crystal violet and lysed. Staining intensity was evaluated photometrically. Adhesion is presented as percentage of seeded cells.

Migration

Cells in the upper part of a Boyden chamber (RPMI 1640/0.1% BSA) were separated from the lower part (RPMI 1640/20% FCS) by 5-μm pore size polycarbonate membranes. After 16 h, cells in the lower chamber were counted. Migration is presented as percentage of input cells.

Proliferation and apoptosis induction

Cells (1 × 10^5) in RPMI 1640/10% FCS were cultured on BSA-, HA (100 μg/ml), or FN (5 μg/ml)-coated plates. Where indicated, cultures contained 2.5–30 μg/ml cisplatin. Apoptosis was determined after 24–72 h. In proliferation assays, 10 μCi/ml [^3]H]thymidine was added after 48 h for an additional 16h.[^3]H]Thymidine incorporation was determined in a beta counter. Mean values ± SD of triplicates are presented.

Cytotoxicity assay

LNCs and SCs were stimulated for 48 h in the presence of 100 U/ml IL-2. Ab-dependent cellular cytotoxicity (ADCC) and NK cell activity was evaluated by [^3]H]thymidine release from labeled (12 h, 10 μCi[^3]H]thy-

Immunoprecipitation and Western blot

Lysates (30 min, 4°C, HEPES buffer, 1% Lubrol, 1 mM PMSF, 1 mM NaVO4, 10 mM NaN3, protease inhibitor mix) were centrifuged (13,000 g, 10 min, 4°C), mixed with Ab (1 h, 4°C), and incubated with protein G-Sepharose (1 h). Washed complexes/lysates, dissolved in Laemmli buffer, were resolved on 10–12% SDS-PAGE. Where indicated, lysates were centrifuged (10 min, 20,000 × g), adjusted to 40% sucrose (4.5 ml), and layered on 1.3 ml 50% sucrose and overlaid with 2.3 ml 30%, 2.5 ml 20%, and 1.3 ml 5% sucrose. After centrifugation (200,000 × g, 16 h), 12 fractions (1 ml) were collected from the top of the tubes. Fractions or pooled fractions were resolved on SDS-PAGE. After protein transfer, blocking, and blotting with Ab, blots were developed with ECL.

Animal experiments

C57BL/6 mice received 1 × 10^6 EL4, E-CD49d, or E-mCD49d, and SCID mice received 1 × 10^5 Jurkat, J-CD44, J-mCD44, or J-trCD44 cells, i.v. or s.c. Where indicated, mice received 100 μg Ab i.v. twice a week, 1 μg cisplatin (1 μg/g body weight) at the indicated time points. Local tumor growth was controlled twice a week. Mice were sacrificed according to the local tumor burden or weight loss. To evaluate tumor cell homing, mice received 1 × 10^7 CFSE-labeled tumor cells i.v. Mice were sacrificed after 48 and 72h. Organs were excised and single-cell suspensions were evaluated for the presence of tumor cells by flow cytometry. Animal experiments were approved by the local government.

Statistics

Significance was evaluated by the two-tailed Student t test (in vivo assays) or the Kruskal–Wallis test (in vitro assays). A p value <0.05 was considered statistically significant.

Results

The adhesion molecule CD44 plays an important role in HSC- and leukemia-initiating cell homing (4). Accordingly, anti-CD44 mobilizes HSCs (17) and drives leukemic cells out of the niche such that they differentiate and die (9). The latter may be due to the engagement of CD44 in signal transduction via associated molecules (46), one of which (i.e., CD49d) also cooperates with CD44 in HSC homing (15). Thus, we asked whether a concomitant blockade of CD44 and CD49d is more efficient in dislodging leukemic cells and promotes leukemia cell apoptosis. To obtain ideas on the underlying mechanisms, leukemia cells were transfected with wt and tail-mutated CD44 or CD49d to prohibit signal transduction.
Characterization of CD49d-transfected EL4 cells and CD44-transfected Jurkat cells

CD44–CD49d cooperation was approached in CD44+ EL4, E-CD49d, and E-mCD49d as well as in CD44− Jurkat, J-CD44, J-mCD44, and J-trCD44. The CD49d Ser198 mutation interferes with CD49d phosphorylation and paxillin and FAK binding (45). The CD44 mutations at position Ser325 and Ser291 prohibit ezrin with CD49d phosphorylation and paxillin and FAK binding (45). The efficacy of the mutations was controlled by coimmunoprecipitation. CD49d coimmunoprecipitates paxillin and FAK in E-CD49d, but not in E-mCD49d lysates; neither mCD44 nor trCD44 coimmunoprecipitates ezrin (Fig. 1D).

Transfection of EL4 with CD49d and of Jurkat with CD44 does not affect additional adhesion molecule and T cell marker expression. EL4 cells express CD18 (high) and ICAMs (low). The CD44− Jurkat subtype strongly expresses CD49c, CD49d, CD50, and CD54 (Supplemental Fig. 1A). EL4 cells express the T cell markers CD4, CD8, and CD25. Jurkat cells express CD4, CD25, CD28, CD69, CD152, and CD154. EL4 cells express TNF ligand; Jurkat cells express CD95 and CD95 ligand. Both lines express CXC4R (Supplemental Fig. 1B). HA or FN stimulation does not affect adhesion molecule or T cell marker expression (data not shown).

Because additional adhesion molecule and T cell marker expression is not affected by EL4 transfection with CD49d or Jurkat transfection with CD44, altered activities of E-CD49d and J-CD44 can be considered to rely on de novo CD49d and CD44 expression, respectively.

CD44 and CD49d engagement in adhesion and migration

CD44 binds HA and the CD49d receptor FN. CD44–CD49d cooperation can strengthen adhesion and migration (47), which was evaluated using HA and FN as stimulus and anti-CD44 (IM7) and anti-CD49d (PS2) for blocking.

Only E-CD49d, but not E-mCD49d, adhered more readily than EL4 to FN and J-CD44, but not J-mCD44 or J-trCD44 to HA and FN (Fig. 2A). HA binding was blocked by IM7; PS2 only blocked mCD44 or mCD49d, into light sucrose gradient fractions (Fig. 3C, Supplemental Fig. 1C). The failure to colocalize could explain the missing cooperation of mutated CD44 and CD49d.

Failed recruitment of mCD44 and mCD49d into “raft-like” domains is due to missing cytokine associations. In EL4 seeded on HA-coated plates, CD44 colocalized with ezrin, ankyrin, and weakly with FAK. In E-CD49d, but not E-mCD49d, colocalization of CD44 with FAK becomes strong, and CD44 also colocalizes with paxillin and CD49d colocalizes with ezrin, ankyrin, FAK, and paxillin (Fig. 3D). In J-CD44, but not J-mCD44 or J-trCD44, CD44 colocalizes with ezrin, ankyrin, FAK, and paxillin (Supplemental Fig. 1D).

Flow cytometry confirmed increased ezrin, src, and FAK phosphorylation in EL4, E-CD49d, and E-mCD49d cells when stimulated by HA cross-linking. However, FN cross-linking only
CD44 AND CD49d COOPERATION IN LEUKEMIA

The impact of concomitant CD44 and CD49d expression on EL4 and Jurkat adhesion and motility. (A and B) Parental and transfected EL4 and Jurkat were seeded (A) in the absence or (B) presence of IM7 or PS2 on BSA-, HA-, or FN-coated plates. Adhesion was determined after 2 h incubation. (C and D) EL4 and Jurkat were seeded in the upper part of a Boyden chamber. The lower chamber contained BSA, HA, FN, or 20% FCS. In (D) cells were preincubated with the indicated Abs. Transwell migration was determined after 16 h. (A and B) The percentage of adherent cells and (C, D) the percentage of migrating cells (mean ± SD, triplicates) are shown. (A and C) *p < 0.05 between parental and transfected cells. (B and D) *p < 0.05 by Ab pretreatment. Coexpression of CD44 and CD49d in leukemia cells promotes adhesion and motility. Inhibition by IM7 and PS2 depends on a function-competent cytoplasmic tail of CD44 and CD49d.

strengthened FAK phosphorylation in E-CD49d. Also, HA crosslinking promoted src and, albeit weakly, FAK phosphorylation only in J-CD44 (Fig. 3E). Finally, IM7 precipitated paxillin and FAK only in E-CD49d and J-CD44 lysates (Fig. 3F). Thus, phosphorylation of the cytoplasmic tail of CD44 and CD49d is required for recruitment into GEM and joint CD44–CD49d interactions with the cytoskeleton.

Having elaborated that CD44 and CD49d promote enhanced adhesion and migration as far as they can communicate via proximity of their cytoplasmic tails with the partner’s adaptor and cytoskeleton linker molecules, we proceede to examine the impact of a joint Ab blockade on leukemia homing and growth in vivo.

The impact of CD44 and CD49d on leukemia cell homing and metastasis

CFSE-labeled E-CD49d cells home more efficiently into spleen, LNs, and BM than do EL4 cells (Fig. 4A). J-CD44 has an advantage in spleen and BM homing but not in LN homing (Fig. 4D). Homing is followed by settlement and growth. Intravenously injected E-CD49d grows in BM, spleen, LNs, and thymus; J-CD44 mostly grows in the BM (Table I). Upon s.c. injection, both lines grow locally, but E-CD49d massively infiltrates spleen and BM (Fig. 4B, 4C, Table II). J-CD44 preferentially metastasizes to the BM also after s.c. application (Fig. 4E, 4F).

To confirm the impact of CD44 and CD49d on homing and settlement, mice received IM7 and/or PS2 concomitantly with EL4. The local growth was not affected or slightly accelerated. Nonetheless, IM7 prolonged the survival time of EL4, E-CD49d−, and E-mCD44−bearing mice. Prolongation of the survival time by PS2 was significant in E-CD49d− but not E-mCD49d−bearing mice. Mice receiving E-CD49d or E-mCD49d and treated with IM7 plus PS2 survived for 47 and 36 d, respectively, and E-CD49d did not grow in two of five mice (Fig. 5A–C).

The prolonged survival time despite unimpaired or pronounced local growth pointed toward IM7 and PS2 interfering with metastatic settlement. In fact, IM7- or PS2-treated mice did not show LN metastases; settlement in the spleen was mostly affected by PS2. Neither IM7 nor PS2 alone sufficed to prevent settlement of E-CD49d in the BM, although no tumor cells were detected 3 wk after application, and the number of tumor cells in the BM was still significantly reduced in IM7− or PS2-treated mice 6 wk after tumor cell application (data not shown). Instead, E-CD49d cells were only recovered in the BM of two of five IM7 plus PS2–treated mice. Application of IM7 plus PS2 did not exert an additive effect on E-mCD49d growth in the BM (Table II), which confirms that the observed therapeutic effect is not exclusively due to a blockade of adhesion.

IM7 and PS2 exerted a similar effect on EL4 growth after i.v. as described for s.c. application. IM7 prolonged the survival time of mice receiving EL4, E-CD49d, or E-mCD49d for 8–12 d in the three groups of mice. PS2 prolonged the survival time of E-CD49d mice for 10 d and of E-mCD49d mice for 8.5 d, which was not significant. Instead, in E-CD49d−bearing mice application of IM7 plus PS2 exerted a significant additive effect with a mean survival time of 54.3 d (Fig. 5D, 5E). Furthermore, significantly fewer leukemia cells were recovered in BM, spleen, and LNs (Fig. 5F). Finally, the percentage of apoptotic EL4 cells was strongly increased in spleen and LNs of mAb-treated mice, but an additive effect of IM7 and PS2 was only seen in E-CD49d (Fig. 5G).

Taken together, IM7 and PS2 hamper BM settlement of i.v. injected and metastatic settlement of s.c. injected EL4. This effect depends on intact CD44 and CD49d, as application of IM7 plus PS2 exerted a stronger effect on E-CD49d settlement in BM and metastatic growth than did either mAb alone, and PS2 more strongly affected metastatic settlement of E-CD49d than did E-mCD49d cells. The latter finding could be in line with apoptosis induction as a consequence of impaired homing and adhesion. In advance of exploring this question, we evaluated the impact of mAb treatment on immune effector cells.
Unexpectedly, expression of activation markers was increased in LNCs of early (2 wk) EL4-, E-CD49d–, and E-mCD49d–bearing mice (Supplemental Fig. 2A) and was hardly affected by mAb treatment (data not shown). mAb treatment also did not affect regulatory T cells in LNCs. Myeloid-derived suppressor cells were expanded in the spleen of IM7-treated mice (Supplemental Fig. 2B, 2C). EL4-, E-CD49d–, and E-mCD49d–bearing mice showed strongly upregulated expression of immunosuppressive as well as immunostimulatory cytokines (Supplemental Fig. 2D), with only IL-2 (IM7 and PS2) and IL-10 expression (IM7) being slightly affected by mAb treatment (data not shown). Lymphocyte proliferation, unimpaired in 2 wk tumor-bearing mice (data not shown), was strongly mitigated in LNCs, although not in SCs, of 4 wk EL4-, E-CD49d–, and E-mCD49d–bearing mice. Nonetheless, poor LNC and unimpaired SC proliferation was promoted by IM7 and PS2 treatment (Fig. 6A). Importantly, NK activity and ADCC were strengthened in mAb-treated mice, with PS2 being effective in E-CD49d– and E-mCD49d–bearing mice (Fig. 6B, 6C). In line with this observation, macrophages (M6) and NK cells were increased in spleen sections of IM7-treated EL4-, E-CD49d–, and E-mCD49d–bearing mice and, slightly less pronounced, in PS2-treated E-CD49d– and E-mCD49d–bearing mice (Fig. 6D).

Because IM7 and PS2 provoked activation of nonadaptive immune effector cells, which equally affected EL4, E-CD49d, and E-mCD49d, native immune effector cell killing cannot explain the pronounced apoptosis induction in IM7 plus PS2–treated E-CD49d (Fig. 5G). Although efficient dislodging of E-CD49d may well contribute to apoptosis induction, the strong effect argues for an additional mAb-initiated apoptotic trigger.

**CD44–CD49d cooperation in leukemia cell proliferation**

CD44 acts as an accessory molecule in T cell activation, with coreceptor functions being initiated by cross-linking via Ab or HA (37). Although IM7 slightly promoted s.c. EL4 growth (Fig. 5A), CD44 or CD49d cross-linking via HA or FN did not affect EL4 or Jurkat proliferation. However, coexpression of CD44 and CD49d (E-CD49d, J-CD44) sufficed for a minor increase in proliferative activity (Supplemental Fig. 3A). Importantly, despite the low efficacy of HA and FN cross-linking, the strong effect argues for an additional mAb-initiated apoptotic trigger.

**CD44–CD49d cooperation in leukemia cell adhesion and motility**

CD44 acts as an accessory molecule in T cell activation, with coreceptor functions being initiated by cross-linking via Ab or HA (37). Although IM7 slightly promoted s.c. EL4 growth (Fig. 5A), CD44 or CD49d cross-linking via HA or FN did not affect EL4 or Jurkat proliferation. However, coexpression of CD44 and CD49d (E-CD49d, J-CD44) sufficed for a minor increase in proliferative activity (Supplemental Fig. 3A). Importantly, despite the low efficacy of HA and FN cross-linking, the strong effect argues for an additional mAb-initiated apoptotic trigger.
tered. Similar findings accounted for J-CD44 that showed strong ERK1/2 activation on HA-coated plates (Supplemental Fig. 3B, 3C). Pronounced lck, MEK1, and p38 phosphorylation of E-CD49d stimulated by CD49d cross-linking via FN was accompanied by pronounced coimmunoprecipitation with CD44 and CD49d (Fig. 7B).

Briefly, coexpression of function-competent CD44 and CD49d supports to a minor degree leukemia proliferation due to pronounced activation of the MAPK pathway via CD44 cross-linking that becomes significantly inhibited by IM7 and PS2.

**CD44–CD49d cooperation in leukemia cell apoptosis resistance**

Blocking HA adhesion can drive leukemic cells into apoptosis. Although cisplatin resistance of EL4 and Jurkat was independent of HA or FN adherence, E-CD49d showed increased apoptosis resistance (Fig. 7C, 7D) indicating CD44–CD49d cooperation. The interpretation was supported by Ab blocking. IM7 and PS2 promoted apoptosis in cells expressing function-competent CD44 and CD49d (Fig. 7E).

Searching for the pathway whereby CD44 or CD49d contributes to apoptosis resistance revealed low caspase-8 activity that was not altered by CD44 and CD49d coexpression, arguing against an impact on receptor-mediated apoptosis. Instead, caspase-9 cleavage and caspase-3 activation was slightly reduced in cisplatin-treated E-CD49d cells. In Jurkat cells, caspase-3 activation and caspase-9 cleavage was mitigated when cells were grown on FN (data not shown) or HA, which, however, was independent of CD44 or mCD44 expression. Thus, in both leukemia lines CD44–CD49d coexpression did not affect or weakly affected caspase expression and activation (Supplemental Fig. 3D, 3E).

Instead, BAD phosphorylation and Bcl2 and Bcl-xL expression were strongly upregulated in E-CD49d, particularly when cross-linked on FN, but proapoptotic BAX and BAK expression were unaltered (Supplemental Fig. 3F, 3G). In J-CD44, HA cross-linking only weakly affected BAD phosphorylation and Bcl-xL expression. However, low BAX expression was further reduced in J-CD44 and J-mCD44 and hardly detectable when cultured on HA-coated plates (Supplemental Fig. 3H). Unexpectedly, PI3K and Akt phosphorylation were not affected in E-CD49d or J-CD44 (data not shown).

In brief, cross-linked CD49d and CD44 protect leukemia cells from apoptosis. However, apoptosis protection via CD44 and

### Table I. Leukemia growth after i.v. injection

<table>
<thead>
<tr>
<th>Leukemia Line (1 x 10^6, i.v.)</th>
<th>Survival Time (d)</th>
<th>Leukemia Cell Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BM</td>
<td>Spleen</td>
</tr>
<tr>
<td>EL4</td>
<td>22.9 ± 3.4</td>
<td>5/5</td>
</tr>
<tr>
<td>E-CD49d</td>
<td>21.1 ± 4.1</td>
<td>5/5</td>
</tr>
<tr>
<td>E-mCD49d</td>
<td>25.8 ± 5.6</td>
<td>5/5</td>
</tr>
<tr>
<td>Jurkat</td>
<td>27.0 ± 6.3</td>
<td>5/5</td>
</tr>
<tr>
<td>J-CD44</td>
<td>24.1 ± 5.4</td>
<td>5/5</td>
</tr>
<tr>
<td>J-mCD44</td>
<td>25.3 ± 7.1</td>
<td>5/5</td>
</tr>
<tr>
<td>J-trCD44</td>
<td>25.5 ± 4.9</td>
<td>5/5</td>
</tr>
</tbody>
</table>

**FIGURE 4.** Impact of CD44 and CD49d coexpression on EL4 cell homing and settlement. (A and D) CFSE-labeled parental and transfected EL4 and Jurkat were injected i.v.; recovery of dye-labeled cells was evaluated after 24, 48, and 72 h in hematopoietic organs, liver, and lung. Mean values of three animals are shown. *p < 0.05 parental and transfected EL4 and Jurkat. (B and C) EL4 and (E, F) Jurkat growth after s.c. injection. Growth rate, mean survival time, and metastatic settlement are shown. *p < 0.05 between parental and transfected EL4 and Jurkat. Coexpression of CD44 and CD49d promotes homing and growth in spleen and BM. Only E-CD49d has an additional advantage in homing and growth in LNs. Although E-CD49d cells show a slightly reduced growth rate, CD44 and CD49d coexpression does not affect the survival time of the leukemia-bearing mice.
CD49d apparently proceed, in part, independently. CD44-associated CD49d promotes activation of antiapoptotic molecules. Because the PI3K/Akt pathway was not affected, BAD phosphorylation likely becomes initiated via MAPKs (48, 49). Instead, downregulation of BAX in CD44-transfected Jurkat was cytoplasmic tail–independent and, thus, unlikely proceeds via CD49d-associated CD44, with the initial trigger being not defined. Nonetheless, in view of Ab-supported apoptosis induction, a blockade of CD44 or CD49d might well strengthen chemosensitivity in vivo.

Anti-CD44 and anti-CD49d support cytotoxic drugs in leukemia therapy

Cisplatin treatment (1 μg/g body weight, i.p.) was started at the day of i.v. tumor cell application and was repeated after 3 and 6 wk. Where indicated, mice received IM7 and/or PS2. Low-dose cisplatin treatment did not significantly prolong the survival time of EL4- and E-CD49d–bearing mice. Instead, when mice concomitantly received IM7, EL4 did not grow in three of five mice and E-CD49d in four of five mice. E-CD49d did not grow in two of five PS2-treated mice and not in IM7 plus PS2–treated mice (Fig. 8A, 8B).

Table II. Leukemia growth after s.c. injection and Ab treatment

<table>
<thead>
<tr>
<th>Leukemia Line</th>
<th>Ab Treatment</th>
<th>Take Rate</th>
<th>Survival</th>
<th>Leukemia Cell Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Time (d)</td>
<td>BM</td>
</tr>
<tr>
<td>EL4</td>
<td>IgG</td>
<td>5/5</td>
<td>21.4 ± 2.9</td>
<td>5/5</td>
</tr>
<tr>
<td>EL4</td>
<td>IM7</td>
<td>5/5</td>
<td>25.8 ± 3.1</td>
<td>5/5</td>
</tr>
<tr>
<td>E-CD49d</td>
<td>IgG</td>
<td>5/5</td>
<td>24.6 ± 4.8</td>
<td>5/5</td>
</tr>
<tr>
<td>E-CD49d</td>
<td>IM7</td>
<td>5/5</td>
<td>40.6 ± 12.7</td>
<td>3/5</td>
</tr>
<tr>
<td>E-CD49d</td>
<td>PS2</td>
<td>5/5</td>
<td>35.8 ± 7.4</td>
<td>4/5</td>
</tr>
<tr>
<td>E-CD49d</td>
<td>IM7 + PS2</td>
<td>3/5</td>
<td>66.2 ± 19.0</td>
<td>1/5</td>
</tr>
<tr>
<td>E-mCD49d</td>
<td>IgG</td>
<td>5/5</td>
<td>22.4 ± 3.6</td>
<td>5/5</td>
</tr>
<tr>
<td>E-mCD49d</td>
<td>IM7</td>
<td>5/5</td>
<td>34.2 ± 4.9</td>
<td>4/5</td>
</tr>
<tr>
<td>E-mCD49d</td>
<td>PS2</td>
<td>5/5</td>
<td>28.8 ± 5.4</td>
<td>5/5</td>
</tr>
<tr>
<td>E-mCD49d</td>
<td>IM7 + PS2</td>
<td>5/5</td>
<td>37.4 ± 5.8</td>
<td>4/5</td>
</tr>
</tbody>
</table>
Leukemic cell settlement was not affected by cisplatin; that is, EL4 and E-CD49d were recovered from BM and spleen, although at a reduced rate. When additionally receiving IM7 or PS2, dispersed tumor cells were recovered in the spleen, but rarely in the BM. In IM7 plus PS2–treated mice, E-CD49d was not recovered in BM or spleen (Fig. 8C). In spleen sections of those cisplatin-treated mice that developed a tumor, small leukemic nodules were recovered. When additionally treated with IM7 or PS2, encapsulated tumor nodules were rare and the majority of the dispersed tumor cells were necrotic, as confirmed by ex vivo staining with annexin V/PI (Fig. 8D, 8E).

To determine whether combined Ab plus chemotherapy suffices to cure established leukemia, cisplatin treatment was started 10 d after i.v. EL4 or E-CD49d application. Although the survival time was not significantly prolonged by delayed cisplatin treatment, one of five EL4-bearing mice receiving in addition IM7 remained tumor-free and the mean survival time was prolonged to 38.8 d (not significant). However, two of five E-CD49d–bearing mice survived when additionally receiving IM7 plus PS2, and the mean survival time was significantly prolonged (Fig. 9A, 9B).

At early stages (2 wk) of tumor growth hematopoiesis was not severely affected by low-dose cisplatin and additional IM7 and PS2 application (Fig. 9C). Recovery of LNCs and SCs was not significantly reduced (data not shown). There was no significant increase in apoptotic lymphocytes in cisplatin-treated mice (Fig. 9D). Low-dose cisplatin did not affect the LNC response to IL-2. Instead, a pronounced response was seen in the presence of IM7 and PS2 (Fig. 9E). In line with the latter finding was the strong ADCC of LNCs from cisplatin plus IM7 and/or PS2–treated mice (Fig. 9F).

Thus, anti-CD44 most efficiently dislodges leukemia cells from the BM but has a weaker effect on settlement in the spleen. Instead, together with anti-CD49d, settlement and growth in the spleen also becomes severely impaired. A joint blockade of CD44 and CD49d additionally supports cytotoxic drug treatment, with the dislodged leukemia cells being no longer protected by their abundantly available natural ligands HA and FN. The direct impact of IM7 and PS2 on leukemia cell homing and survival is supported by ADCC, with low-dose cisplatin not affecting hematopoiesis or mature leukocytes.

**Discussion**

CD44 contributes to HSC- and leukemia-initiating cell homing (4, 50), the feature of which is used for HSC mobilization (17, 51) and leukemia-initiating cell dislodgment by anti-CD44, driving the latter into differentiation and apoptosis (5, 6). Because anti-CD49d also promotes HSC and leukemia cell mobilization (16, 52), and a CD44–CD49d cooperation additionally supports proliferation and apoptosis resistance (23, 53), we assumed that a blockade of both CD44 and CD49d may be a more efficient therapeutic. To control the hypothesis and to unravel the underlying mechanism, we compared joint activities of signaling-competent versus -deficient CD44 and CD49d in leukemia. We demonstrate that the efficacy of a blockade of CD44 can be significantly improved by concomitantly blocking signaling-competent CD49d without attacking hematopoiesis or leukocyte activation.

**Contribution of CD44 and CD49d to leukemia growth and metastasis**

Recovery of dye-labeled EL4 and Jurkat shortly after i.v. injection revealed CD44 supporting homing into BM and spleen and CD49d facilitating BM, spleen, and LN homing. The impact of CD44 and CD49d on BM homing was confirmed by leukemia growth after
s.c. injection. EL4 and E-mCD49d metastasize in BM and spleen and E-CD49d additionally in LNs and thymus. Jurkat cells metastasize in BM and spleen, with the latter being supported by CD44 expression. The impact of CD44 and CD49d on metastatic leukemia growth became particularly obvious upon IM7 and PS2 treatment. Despite Ab treatment not preventing local tumor growth, the survival time of Ab-treated mice was prolonged owing to impaired settlement in BM and spleen. Only in E-CD49d–bearing mice did the survival time become significantly prolonged when treated with IM7 plus PS2 compared with mice receiving either IM7 or PS2, and in two of five mice E-CD49d cells were not recovered in BM and spleen at autopsy.

Additionally, a strong increase in apoptotic leukemia cells was only seen in IM7 plus PS2–treated E-CD49d, but not E-mCD49d, metastases. This finding suggests that in vivo CD44 and CD49d, besides promoting homing and migration, have a strong impact on leukemia cell apoptosis resistance. The latter was further supported by concomitant cisplatin and IM7 or PS2 treatment. Low-dose cisplatin hardly sufficed for apoptosis induction in EL4, but apoptosis of E-CD49d was strongly upregulated in cisplatin and IM7 or PS2–treated mice, and E-CD49d did not grow in cisplatin and IM7 plus PS2–treated mice. Finally, IM7 did not suffice to significantly prolong the survival time of mice receiving low-dose cisplatin after EL4 settlement. Instead, E-CD49d did not grow in two of five mice and the survival time was significantly prolonged in mice receiving IM7 plus PS2.

Importantly, too, there was a striking increase in Mφ and NK cells infiltrating leukemic pseudofollicles. Both IM7 and PS2 supported NK activation and ADCC in E-CD49d– and E-mCD49d–bearing mice.

We discuss below why anti-CD44 together with anti-CD49d exert a stronger therapeutic effect than either Ab alone and outline our viewpoint on stimulation of the nonadaptive immune system by anti-CD44 or anti-CD49d.

**CD44/CD49d complex and leukemia cell adhesion and migration**

Anti-CD44 together with anti-CD49d most efficiently interferes with leukemia cell homing into the BM and metastatic settlement. In vitro, leukemic cells expressing CD44 and CD49d have an advantage in adhesion to HA and FN (26, 54–56). However, adhesion promotion requires an intact cytoplasmic tail of both molecules. Cytoplasmic tail-mutated CD44 or CD49d exert no effect, and Abs only inhibit the intact molecules’ activity. This excludes a higher number of receptors to account for pronounced adhesion and transwell migration. In line with our interpretation of
CD44 activation via CD49d and vice versa, only intact CD44 and CD49d colocalize and coimmunoprecipitate, which allowed for CD49d-associated paxillin and FAK activation (54, 57) via CD44 and for CD44-associated src and ezrin phosphorylation (35, 36) via CD49d (23, 58, 59). Thus, activated, HA-binding CD44 can drive CD49d into a motility-promoting complex (54), and activated CD49d supports exchange of the serine phosphorylation in the cytoplasmic tail of CD44 (33) allowing for ezrin binding and linkage to the actin cytoskeleton (34).

These in vitro studies are in line with the in vivo growth profile of EL4 and Jurkat and explain the superior effect of anti-CD44 plus anti-CD49d on E-CD49d and J-CD44.

CD44, CD49d, and leukemia cell proliferation and apoptosis resistance

The association of CD44 with CD49d exerts a strong effect on leukocyte proliferation, being mostly triggered via lck phosphorylation and subsequent MAPK pathway activation (23). Instead, the autonomous growth of E-CD49d and I-CD44 was only slightly supported when seeded on HA or FN. However, particularly E-CD49d proliferation on FN was strongly inhibited in the presence of IM7 or PS2. Signaling via CD44 and CD44-associated CD49d proceeds via the MAPK pathway without a measurable contribution by the JNK pathway. In malignant B lymphocytes CD49d initiates activation of ERK1/2 via Zap70 phosphorylation (40). Where precisely CD44- or CD49d-initiated signals join in T leukemia has not been explored. Irrespective of this open question, the strong blockade of leukemia proliferation by IM7 plus PS2 will contribute to the therapeutic efficacy in vivo.

Leukemia cells are rather apoptosis-resistant but are efficiently driven into apoptosis by anti-CD44 and anti-CD49d, particularly in combination with low-dose cisplatin, where the strongest effect is seen in anti-CD44 plus anti-CD49d-treated E-CD49d. An in vitro analysis confirmed increased apoptosis resistance of E-CD49d and
J-CD44 only upon ligand binding, where IM7 and PS2 interfered with apoptosis resistance. Trying to unravel the engaged signaling pathway revealed that CD44-associated CD49d cross-linking is accompanied by pronounced BAD phosphorylation. Because PI3K and Akt phosphorylation (60, 61) remained unaltered, it is likely that phosphorylated ERK1/2 directly promotes BAD phosphorylation (48, 49), which implies cooperativity of CD49d with CD44 proceeding similarly in apoptosis resistance and proliferation. In Jurkat, CD44 cross-linking mostly promoted downregulation of proapoptotic BAX (62), which was independent of the association with CD49d. The pathway whereby CD44 activation initiates BAX downregulation remains to be explored.

Taken together, the in vitro findings of joint activities of CD44 and CD49d on apoptosis resistance cannot fully explain the striking apoptosis induction in cisplatin-treated E-CD49d–bearing mice that received IM7 plus PS2. Thus, we suggest that the important and dominating event is the Ab-induced dislodging, which drives the leukemic cell into apoptosis, particularly when concomitantly exposed to a cytotoxic drug.

Hematopoiesis and leukocyte activation in anti-CD44 and anti-CD49d–treated mice

At early stages of tumor growth, EL4 cells were not immunosuppressive and rather supported leukocyte activation with comparable upregulation of immunostimulatory and immunosuppressive cytokines. Regulatory T cells were not affected and myeloid-derived suppressor cells only were slightly expanded in IM7-treated mice. Additionally, particularly in metastatic tissue there was a striking increase in MΦ and NK cells infiltrating leukemic pseudofollicles. Both IM7 and PS2 supported NK activation and ADCC in E-CD49d– and E-mCD49d–bearing mice. Importantly, too, low-dose cisplatin did not interfere with leukocyte responsiveness and, at early stages of tumor growth, hematopoiesis was not impaired, and LNCs and SCs were not driven into apoptosis by low-dose cisplatin. Instead, the proliferative responses to IL-2 as well as ADCC were stimulated by Ab treatment.

FIGURE 9. The impact of anti-CD44/anti-CD49d treatment on EL4 growth under therapeutic cisplatin treatment. Mice received an i.v. injection of EL4 or E-CD49d and twice a week IM7 or IM7 plus PS2. At days 10, 31, and 52 mice received i.p. injections of 1 μg/g body weight cisplatin. (A) Survival time, survival rate, and (B) means ± SD survival time. Significant differences to mice not receiving cisplatin or IM7/PS2 are indicated. (C) Recovery of HSCs in the BM 3wk after EL4 application (means ± SD, triplicates, flow cytometry). (D) Annexin V/PI-stained LNCs and SCs 3 wk after tumor cell application (means ± SD, triplicates, flow cytometry). (E) [3H]thymidine incorporation by LNCs cultured in the presence of IL-2 and IM7, PS2, or IM7 plus PS2 as in vivo (means ± SD, triplicates). (F) ADCC evaluated after ex vivo culture (48 h, 100 U/ml IL2) using EL4 or E-CD49d as targets and control IgG, IM7, PS2, or IM7 plus PS2 as in vivo. Percentage cytotoxicity (means ± SD, triplicates) is shown. (E and F) *p < 0.05 by Ab treatment; †p < 0.05 between EL4 and E-CD49d. The impact of cisplatin becomes weaker when started after EL4 settlement in the BM. However, cisplatin together with anti-CD44 and anti-CD49d still suffice for a significant prolongation of the survival time of E-CD49d–bearing mice. Hematopoiesis was not impaired, and LNCs and SCs were not driven into apoptosis by low-dose cisplatin. Instead, the proliferative responses to IL-2 as well as ADCC were stimulated by Ab treatment.
Finally, we note that unimpaired hematopoiesis and mature leukocyte activity were only seen in low-dose cisplatin-treated mice. Increasing the cisplatin dose promoted strong side effects, particularly in mice concomitantly receiving IM7, where anti-CD44 was repeatedly reported to affect nontransformed cells under poor health conditions (46, 63). Furthermore, though anti-CD44 apparently does not significantly affect steady-state hematopoiesis, it becomes a serious danger during reconstitution (64).

As reported by Avin et al. (65) for the therapy of a B cell lymphoma, a bispecific Ab targeting CD44 and a B leukemia marker circumvents side effects. It more efficiently disrupts the communication between the leukemia cell and the host and still stimulates a nonadaptive immune response. This may also account for an anti-CD44/anti-CD49d bispecific Ab. To the extent that the leukemic cell expresses a CD44 variant isoform, side effects could be further minimized using a CD44 variant isoform specific Ab.

In conclusion, the therapeutic effect of mAbs frequently is based on activation of natural immune defense mechanisms (66, 67). This also accounted for anti-CD44 and anti-CD49d. However, the high therapeutic efficacy is due to anti-CD44 and anti-CD49d directly attacking the leukemic cell. Most prominent is the interference with homing, settlement, and motility, where joint signaling plays an important role. Furthermore, leukemia proliferation and drug resistance were severely impaired by an Ab blockade and, upon in vivo dislodging by anti-CD44/anti-CD49d, leukemia cells expressing the CD44/CD49d complex were efficiently driven into apoptosis. Thus, owing to the cooperation of CD44 and CD49d in leukemia homing, growth, and progression, a joint blockade of both molecules is a potent therapeutic in combination with low-dose chemotherapy. For clinical translation we suggest an anti-CD44/anti-CD49d bispecific Ab to guarantee concomitant targeting of CD44 and CD49d to optimally complement chemotherapy.

Acknowledgments

We cordially thank Dr. Rachid Marhaba for most helpful discussions and collaboration with αCD44 integrins. Blood 107: 3503–3510.


References


