

Homing of Human B Cells to Lymphoid Organs and B-Cell Lymphoma Engraftment Are Controlled by Cell Adhesion Molecule JAM-C

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Abstract

Junctional adhesion molecule C (JAM-C) is expressed by vascular endothelium and human but not mouse B lymphocytes. The level of JAM-C expression defines B-cell differentiation stages and allows the classification of marginal zone-derived (JAM-C-positive) and germinal center-derived (JAM-C-negative) B-cell lymphomas. In the present study, we investigated the role of JAM-C in homing of human B cells, using a xenogeneic nonobese diabetic/severe combined immunodeficient mouse model. Treatment with anti-JAM-C antibodies in short-term experiments reduced migration of normal and malignant JAM-C-expressing B cells to bone marrow, lymph nodes, and spleen. Blocking homing to the spleen is remarkable, as most other antiadhesion antibodies reduce homing of B cells only to bone marrow and lymph nodes. Long-term administration of anti-JAM-C antibodies prevented engraftment of JAM-Cpos lymphoma cells in bone marrow, spleen, and lymph nodes of mice. Plasmon resonance studies identified JAM-B as the major ligand for JAM-C, whereas homotypic JAM-C interactions remained at background levels. Accordingly, anti-JAM-C antibodies blocked adhesion of JAM-C-expressing B cells to their ligand JAM-B, and immunofluorescence analysis showed the expression of JAM-B on murine and human lymphatic endothelial cells. Targeting JAM-C could thus constitute a new therapeutic strategy to prevent lymphoma cells from reaching supportive microenvironments not only in the bone marrow and lymph nodes but also in the spleen. *Cancer Res*; 73(2); 640–51. ©2012 AACR.

Introduction

Despite new treatment strategies, most mature B-cell lymphomas still remain incurable. Evidence suggests that stromal cells in specialized tissue microenvironments, such as bone marrow and secondary lymphoid organs, are essential for disease progression. In fact, contact with stromal cells and the cytokines secreted by them favor viability, differentiation, proliferation, and retention of B cells and provide protection from conventional chemotherapy (1, 2).

To enter lymphoid organs, B cells must adhere to endothelium and transmigrate across the endothelial barrier, thus chemokines and adhesion molecules are important in the homing of normal and malignant B cells and in lymphoma dissemination (3–10). Both firm adhesion and transmigration

across endothelial barriers depend on the ability of circulating cells to interact with endothelium through selectin ligands, integrins, or CD44. However, except CD44, these molecules are not involved in homing to the spleen.

Junctional adhesion molecule C (JAM-C) belongs to the immunoglobulin (Ig) superfamily and is composed of 2 extracellular Ig-like domains and a cytoplasmic tail with a PDZ-binding motif (11). JAM-C has been described as an endothelial adhesion molecule localized at tight junctions and expressed by high endothelial venules (HEV) and lymphatic vessels in lymphoid organs. JAM-C expression has also been described in human hematopoietic cells, for example, platelets, activated T cells, and natural killer (NK) cells (12), and our group has previously shown that JAM-C is expressed on the surface of normal and malignant B cells. Its differential expression distinguishes memory germinal center B cells (CD27pos, JAM-Cneg) from memory non-GC B cells (CD27pos, JAM-Cpos; ref. 13). The expression of JAM-C in different B-cell lymphomas allowed the classification into 2 types of B-cell malignancies: JAM-Cneg [chronic lymphocytic leukemia (CLL), follicular lymphoma, and diffuse large B-cell lymphoma (DLBL)] and JAM-Cpos [marginal zone B-cell lymphoma (MZBL) and hairy cell leukemia (HCL)] lymphomas.

Several interactions for JAM-C have been described as: homophilic JAM-C/JAM-C and heterophilic JAM-C/MAC-1 (α M β 2) or JAM-C/JAM-B interactions (11). Among these, JAM-B seems to be the major ligand for JAM-C (14, 15). Thus,

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JAM-C on human leukocytes could interact with vascular JAM-B to mediate leukocyte adhesion and transmigration.

In the current study, we analyzed the *in vivo* migratory behavior of normal human B lymphocytes and pathological B cells from JAM-Cpos B-cell lymphomas. As murine B cells do not express JAM-C and the aim of this study was to investigate human B-cell lymphomas, we used adoptive transfer of human B cells into immune deficient nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice to conduct short-term homing assays. This model is widely used for the study of human B-cell migration *in vivo*, as the major endothelial integrin ligands, chemokines, selectins, and selectin ligands of lymphoid organs are highly conserved between human and mouse (3, 16, 17). We report differential migration of peripheral blood JAM-C-expressing lymphomas [mantle cell lymphoma (MCL) and MZBL] to lymphoid organs, compared with normal B cells. Using anti-JAM-C antibodies, the homing process for normal B cells as well as for human primary lymphoma cells was impaired, most remarkably the homing to the spleen. In addition, we show that administration of anti-JAM-C antibodies impaired significantly long-term engraftment and lymphoma development in NOD/SCID mice. These data describe for the first time an involvement of JAM-C in the B-cell traffic to bone marrow, lymph nodes, and spleen, as well as in B-cell lymphoma growth.

Materials and Methods

Human samples and cell lines

Human samples were obtained after informed consent and used according to the procedures approved by the local ethics committee and the Declaration of Helsinki. Peripheral blood was obtained from healthy donors and from patients with leukemic B-cell lymphomas. Mononuclear cells were collected following separation on Ficoll-Paque (GE-Healthcare). B cells were enriched by negative selection using a human B-cell enrichment kit (EasySep, StemCell Technologies), according to manufacturer's instructions.

The human cell lines Jeko-1 (MCL) and K1718 (MZBL) were a kind gift from J.A. Martinez-Climent (Laboratory of Molecular Oncology, Center for Applied Medical Research, University of Navarra, Madrid, Spain). The cell lines Raji, MDCK, CHO, and K562 were purchased from the American Type Culture Collection. Human umbilical vein endothelial cells (HUVEC) were produced in our laboratory and used during first passages in culture. Phenotype of cell lines was tested and authenticated by flow cytometry before use.

Transfection of JAM-Cneg cell lines

The JAM-Cneg cell lines RAJI, MDCK, CHO, and K562 were cultured in RPMI supplemented with 10% fetal calf serum, antibiotics, glutamine, amino acids, and sodium pyruvate. Cells were stably transfected using Amaxa Nucleofection System according to manufacturer's instructions with a pcDNA plasmid encoding a neomycin-resistance cassette without (=pcDNAe) or with full-length human JAM-C cDNA (=HuJAM-C; ref. 13). After 2 weeks selection with G418, 1 mg/mL (Invitrogen) cells were fluorescence-activated

cell sorting (FACS)-sorted and further expanded under G418 selection (600 µg/mL). JAM-C surface expression was confirmed by FACS analysis before each experiment.

Cell adhesion assays

NUNC 269787 adhesion plates were successively coated with anti-huFc antibody (Jackson ImmunoResearch) at 2.5 µg/mL, Fc-labeled proteins at 2.5 µg/mL, and blocked with 1% bovine serum albumin (BSA; Sigma). Cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) as recommended by the manufacturer (Invitrogen) and Fc-receptors were blocked (Fc-blocking solution; Miltenyi Biotech). Cells were then incubated in Dulbecco's Modified Eagle's Medium with rabbit IgG or anti-human JAM-C affinity-purified polyclonal antibody for 15 minutes at room temperature. Finally, 10^5 to 5×10^5 cells were added per well and incubated for 30 minutes in a CO₂ incubator at 37°C. Then wells were carefully washed with PBS before adding 150 µL of 1% SDS in PBS for at least 4 hours with agitation at room temperature. 100 µL of lysed cells were transferred into a 96-well plate, (micro-clear plates black, Greiner) and fluorescence (λ_{ex} 485 nm; λ_{em} 538 nm) was measured with a Flex Station Microplate reader (Molecular Devices). Data are expressed either in arbitrary fluorescence units or as the ratio of the fluorescence measured on JAM-B Fc-coated wells to the fluorescence measured on wells coated with Fc control protein.

Binding assays of soluble proteins

Different cell types expressing different levels of JAM-C were used for binding assays, including: Raji pcDNA (mock-transfected control) and K1718 as JAM-Cneg cells; Raji HuJAM-C X, Raji HuJAM-C Y (2 different batches of JAM-C-transfected cells), Jeko-1, HUVEC, JAM-C-transfected MDCK, CHO, and K562 as JAM-Cpos cells. Cells were incubated with either recombinant human IgG1 Fc (R&D) as a control or human JAM-B Fc (R&D) at 1 µg/mL for 60 minutes at 4°C in PBS-BSA, 2×10^5 cells per well. In blocking experiments, cells were preincubated with anti- $\alpha 4$ integrin antibody (1 µg/mL, clone HP2/1, AbD Serotec) or anti-human JAM-C affinity-purified polyclonal antibody. After washing, cells were incubated for 60 minutes at 4°C with DyLight 488 goat anti-human IgG1Fc fragment [F(ab), affinity-purified (Jackson Immunoresearch)] and analyzed on a FACScalibur (BD Biosciences). Mean fluorescence values were calculated with Cellquest software and data expressed as the ratio of JAM-B Fc mean fluorescent intensity (MFI) to Fc control MFI.

Surface plasmon resonance

Interaction studies were conducted on a Biacore 2000 (GE Healthcare). Recombinant proteins were immobilized at a concentration of 5 µg/mL on a CM5 sensor chip using the Amine Coupling Kit (NHS-EDC) provided by the Biacore supplier. Background signal from a reference channel without soluble JAM-C was automatically subtracted. Proteins (30 µL) were injected (Kinject) in running buffer (Tris-HCl 400 µmol/L pH 7.4, containing 145 mmol/L NaCl) at a flow rate of 20 µL/min.

Mice

NOD.CB17-Prkdcscid/J (NOD/SCID) mice were bred under defined flora conditions at the University Medical Center, University of Geneva (Geneva, Switzerland), in sterile micro isolator cages. All experiments were approved by the Animal Care Committee of Geneva and by the Swiss National Veterinary Law.

B-cell homing assays

Human B cells obtained from healthy donors or from patients with a leukemic B-cell lymphoma were injected into the tail vein of nonirradiated 4- to 8-week-old NOD/SCID mice ($4\text{--}20 \times 10^6$ cells per mouse). For blocking experiments, B cells were incubated 30 minutes before injection with rabbit- (13) or goat- (R&D) anti-JAM-C affinity-purified antibodies ($10 \mu\text{g}/\text{mL}$), with anti- $\alpha 4$ integrin antibody ($0.5 \mu\text{g}/\text{mL}$, clone HP2/1) or a combination of both. Polyclonal anti-JAM-C antibodies were used to ensure maximal blocking of epitopes on the JAM-C antigen. In control experiments, B cells were incubated with either rabbit IgG, goat IgG, a combination of rabbit and mouse IgG, or rabbit anti-human CD19 (Abgent), as an irrelevant binding antibody.

One hour after injection, mice were sacrificed and blood, liver, bone marrow, spleen, and lymph nodes (mesenteric, inguinal, brachial, axillary, and cervical) were collected. Human B cells from the different organs were detected by flow cytometry using human-specific anti-CD45 and anti-CD19 antibodies and quantified by normalizing the number of B cells detected in 10^6 cells acquired by FACS, per 10^6 of injected B cells. Noninjected mice were used as controls. Supplementary Fig. S1A shows that the quantity of injected B cells (between 4 and 20×10^6 cells were used per injection) did not alter their homing behavior. Supplementary Fig. S1B shows that preincubation of cells with rabbit anti-human CD19 did not alter the detection of B cells.

Long-term assays in NOD/SCID mice

Jeko-1 cells (5×10^6 cells) were injected intravenously into 4- to 8-week-old NOD/SCID mice. Twenty-four hours after injection, mice were treated with either Rabbit IgG (control group) or anti-JAM-C antibody, $10 \mu\text{g}/\text{mouse}$. Subsequently, antibodies were administered intravenously 2 times per week for 3 weeks. Mice were monitored for general condition and weight loss, and sacrificed at day 24. Blood, liver, bone marrow, spleen, and lymph nodes were collected and analyzed for the presence of Jeko-1 cells by flow cytometry using human-specific anti-CD45 and anti-CD19 antibodies.

Immunofluorescence staining of lymphoid organs

Five-micrometer cryosections were obtained from spleen and lymph nodes of injected mice and from human tonsils. Sections were fixed in cold methanol-acetone for 5 minutes, dried, and rehydrated in 0.1% BSA-PBS for 15 minutes at room temperature. Mouse samples were incubated for 1 hour with rabbit anti-mouse JAM-B affinity-purified polyclonal antibody (1/100, obtained after immunization with murine JAM-B-FLAG fusion protein) and rat anti-mouse PECAM-1 antibody GC51 ($1 \mu\text{g}/\text{mL}$; ref. 18) or mouse anti-human CD20 antibody (clone

L26, Dako). Tonsil sections were incubated with rabbit anti-human JAM-B affinity-purified antibody (1/100) and mouse anti-human VE-CAD 55-7H1 (BD Biosciences). Sections were washed 3 times for 5 minutes at room temperature and incubated for 1 hour with DyLight 649 F(ab)2 donkey anti-rabbit, DyLight 488 F(ab)2 donkey anti-rat (Jackson Laboratories), and human serum-adsorbed DyLight 488 goat anti-mouse antibodies (Kirkegaard & Perry Laboratories). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; $200 \text{ ng}/\text{mL}$, Invitrogen), slides were mounted in Moewiol (Sigma-Aldrich), and fluorescence was scanned on LSM 510Meta confocal microscope (Carl Zeiss).

Results

We have previously shown that JAM-C is expressed on human B lymphocytes during 2 well-defined differentiation stages: mature CD27^{neg} naïve B cells and CD27^{pos} memory B cells. These cells constitute the major populations of circulating B cells in the peripheral blood compartment. Because JAM-C plays an important role in neutrophil and monocyte migration (19, 20), we investigated whether JAM-C could also influence the migration of circulating B cells to lymphoid organs.

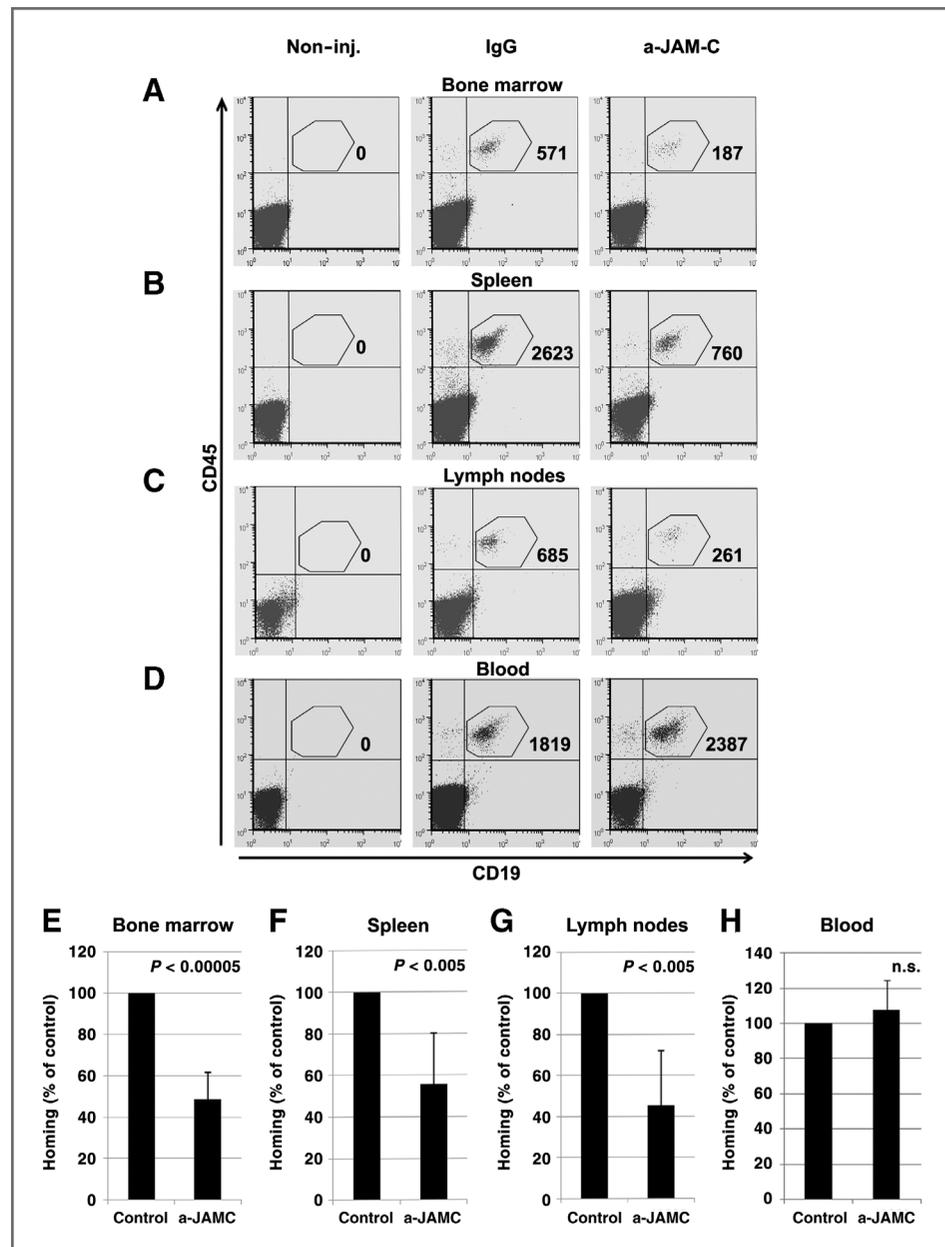
Homing of normal human B cells to lymphoid organs is blocked by anti-JAM-C antibodies

Human B cells from peripheral blood of healthy donors were incubated with affinity-purified rabbit anti-JAM-C antibody or control IgG and injected into the tail vein of NOD/SCID mice. One hour postinjection, B cells were quantified in bone marrow, spleen, lymph nodes, and blood by flow cytometry, using human-specific anti-CD45 and anti-CD19 antibodies (Fig. 1A–D). Affinity-purified rabbit anti-JAM-C antibodies specifically decreased the homing of B cells to bone marrow, spleen, and lymph nodes but did not alter the number of B cells in circulation (Fig. 1). Similar results were obtained by treatment of B cells with affinity-purified goat anti-JAM-C antibody (data not shown), whereas preincubation of B cells with rabbit anti-human CD19 antibody did not influence the homing (Supplementary Fig. S1C), showing the specific effect of anti-JAM-C antibodies.

To study the localization of migrated human B cells in lymphoid organs, tissue sections were stained with the B-cell marker anti-CD79. In accordance to the results obtained from flow cytometry, a reduced number of B cells were observed in the tissue sections when cells were incubated with anti-JAM-C antibodies (Supplementary Fig. S2).

To exclude any possible effects of anti-JAM-C antibody binding on B-cell viability, cells were cultured during 1 hour in the absence or presence of antibodies. No differences in the percentages of apoptotic or dead cells were detected between anti-JAM-C-treated and control cells (Supplementary Fig. S3). To further exclude any indirect effects of antibody treatment, we compared expression of activation markers CD69 and CD38, chemokine receptor CXCR4, adhesion molecules CD44, JAM-A, and the integrin subunits α -M, α -6, β -2, α -4, and β -7 on anti-JAM-C-treated and on control B cells. No significant differences in expression were observed for any of the markers

Figure 1. Homing of normal human B cells to lymphoid organs is reduced by anti-JAM-C antibodies. A–D, human B cells ($4\text{--}20 \times 10^6$), either incubated for 30 minutes with control IgG or with anti-JAM-C antibody, were injected into NOD/SCID mice. One hour after injection the mice were sacrificed and bone marrow, spleen, lymph nodes (mesenteric, inguinal, brachial, axillary, and cervical), and blood were analyzed by FACS for the presence of human B cells using anti-CD19 and anti-CD45 antibodies. Results of 1 representative experiment are shown. Gates indicate the number of human B cells recovered from bone marrow (A), spleen (B), lymph nodes (C), and blood (D), expressed as number of detected B cells per 10^6 cells acquired by FACS per 10^6 B cells injected into the mice. E–H, effect of anti-JAM-C antibody on the homing of human B cells to bone marrow (E), spleen (F), lymph nodes (G), and blood (H), relative to control (control = 100%). Data are expressed as mean \pm SD from 7 independent experiments, 2 mice per experiment. Differences in homing between antibody-treated and control cells were analyzed using Student *t* test, $P < 0.05$.



examined (Supplementary Fig. S4). To show the specificity of the anti-JAM-C antibody, additional experiments were carried out using JAM-Cneg B cells. Anti-JAM-C treatment did not alter the homing pattern of these cells (Supplementary Fig. S5A).

As homing of B cells to all the lymphoid organs studied was decreased, we investigated whether B cells could have been redirected to other organs. An increase in the number of B cells in liver of anti-JAM-C-treated mice compared with control was found (Supplementary Fig. S5B).

Homing of B cells was also analyzed on the basis of the presence or absence of JAM-C on the B-cell surface rather than on antibody blocking of the JAM-C molecule. Peripheral blood B cells from normal blood donors ($\sim 70\%$ JAM-Cpos cells, 30%

JAM-Cneg cells; ref. 13) were injected into mice and human B cells recovered from the organs were stained with anti-huJAM-C antibodies. Percentages of JAM-Cpos and JAM-Cneg B cells were quantified. As shown in Supplementary Fig. S5C, a majority of JAM-Cpos cells migrated to bone marrow, spleen, and lymph nodes compared with liver, the organ in which anti-JAM-C antibody treatment had no inhibitory effect (Supplementary Fig. S5B).

Taken together, these data suggest a major and specific role of JAM-C in the homing of normal B lymphocytes to bone marrow, spleen, and lymph nodes.

Because $\alpha 4$ integrin (VLA-4) is one of the most important adhesion molecules implicated in B-cell homing (3, 10), we compared the effects of anti-JAM-C antibody treatment with

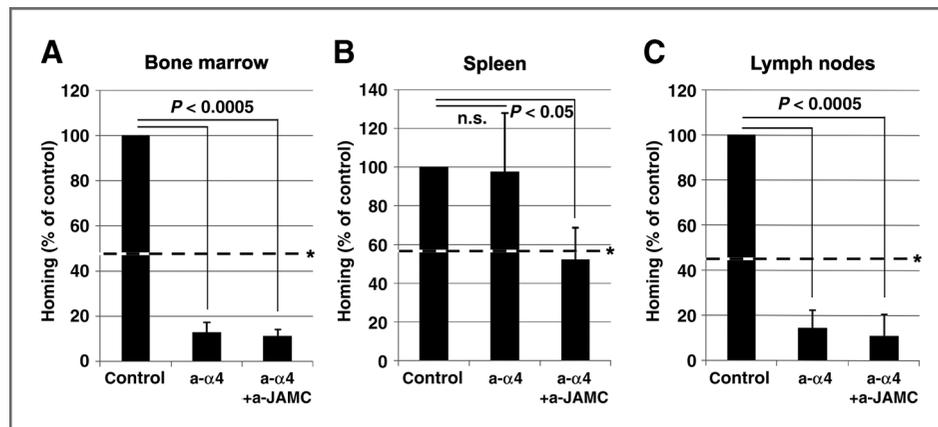


Figure 2. Homing of normal human B cells is inhibited by a combination of anti-JAM-C and anti- $\alpha 4$ integrin antibodies. Human B cells ($4-20 \times 10^6$) treated for 30 minutes with control IgG, with anti- $\alpha 4$ integrin antibody, or with a combination of anti- $\alpha 4$ integrin and anti-JAM-C antibodies were injected into NOD/SCID mice. One hour after injection the mice were sacrificed and bone marrow, spleen, and lymph nodes were analyzed by flow cytometry for the presence of human B cells using anti-CD19 and anti-CD45 antibodies. Data show percentage of human B cells that homed to bone marrow (A), spleen (B), and lymph nodes (C), relative to control (control = 100%). Data correspond to mean \pm SD of 4 independent experiments, 3 mice per experiment. Differences in homing between antibody-treated and control cells were analyzed using ANOVA, followed by Tukey *post hoc* analysis, $P < 0.05$. *, average inhibition of homing with a-JAM-C antibody.

anti- $\alpha 4$ integrin antibody treatment or to a combination of anti-JAM-C and anti- $\alpha 4$ antibodies. Anti- $\alpha 4$ integrin antibody decreased homing to bone marrow and lymph nodes to a greater extent than anti-JAM-C alone, but had no effect on homing to the spleen. Interestingly, the combination of both antibodies resulted in inhibition of homing to all 3 lymphoid organs (Fig. 2).

Homing of malignant lymphoma B cells to lymphoid organs is blocked by anti-JAM-C antibodies

Because we showed that JAM-C is involved in normal B lymphocyte homing, we next investigated whether migration of malignant lymphoma B cells to lymphoid organs could also be impaired by anti-JAM-C antibodies. We had previously reported that the expression of JAM-C in B-cell lymphomas revealed a disease-specific pattern, distinguishing JAM-Cpos

from JAM-Cneg B-cell lymphomas (13). In Fig. 3, an updated and extended version of this analysis for 163 B-cell lymphoma cases is shown. The frequency of JAM-Cpos cases for each pathology confirmed our previous data, with the exception of MCL, in which the analysis of a larger patient group showed a majority of JAM-Cpos cases (15/21). Therefore, JAM-C expression clearly allows the distinction between JAM-Cpos lymphomas (MZBL, MCL, and HCL) and JAM-Cneg lymphomas (CLL and follicular lymphoma).

B cells from patients with JAM-Cpos or JAM-Cneg lymphomas as well as from healthy donors were injected into NOD/SCID mice (Fig. 4A). Blood, bone marrow, spleen, and lymph nodes were analyzed 1 hour after injection. The homing of JAM-Cpos lymphoma B cells to lymph nodes was reduced by 77% as compared with normal B lymphocytes, whereas no difference in migration to bone marrow or spleen was observed

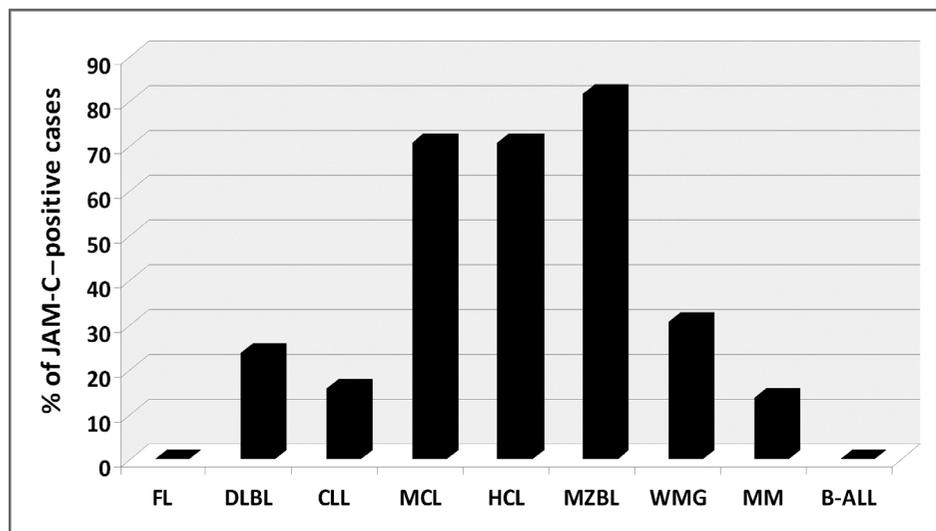
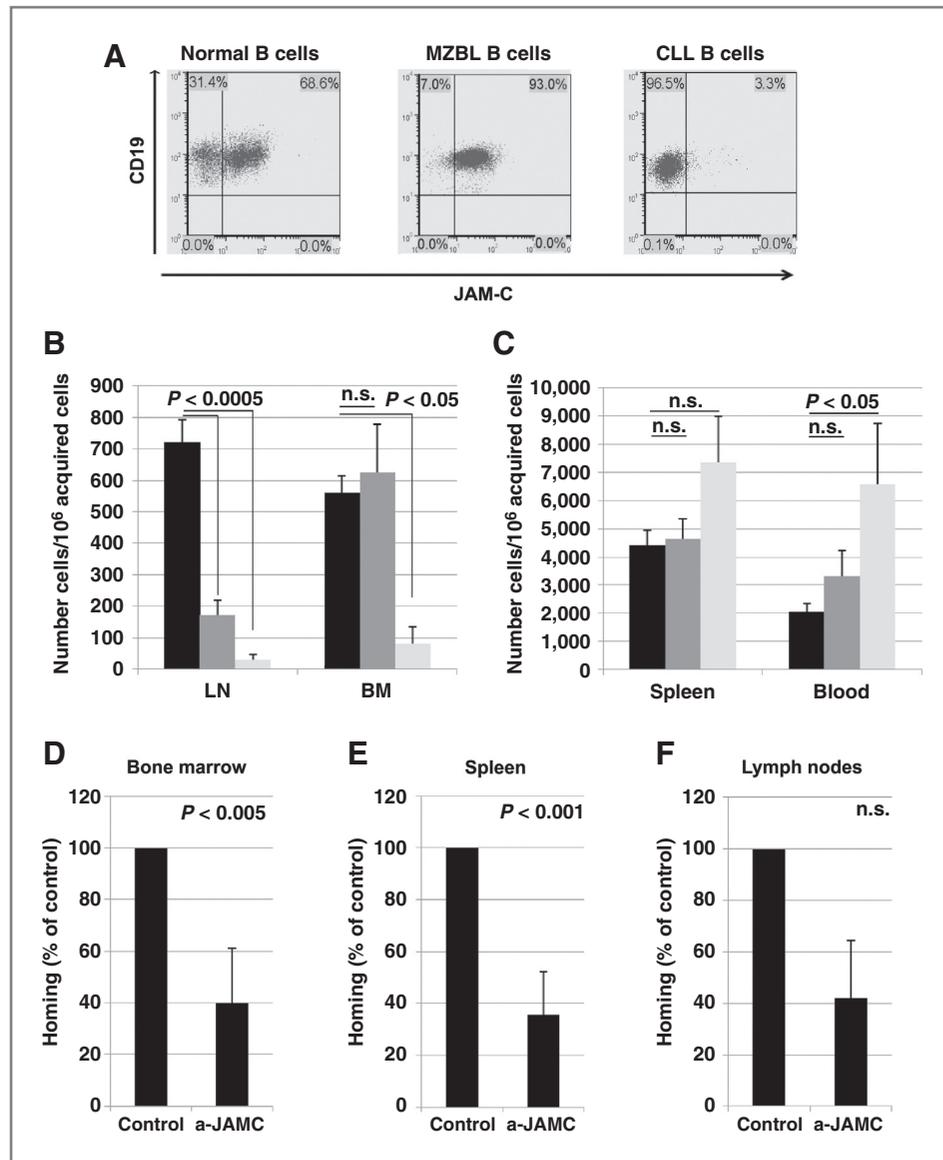


Figure 3. JAM-C expression by human B-cell lymphomas. Different types of human B-cell lymphomas were studied by flow cytometry for the expression of JAM-C: 8 follicular lymphoma (FL), 17 DLBL, 44 CLL, 21 MCL, 7 HCL, 38 MZBL, 16 WM (Waldenstroem's macroglobulinemia), 7 MM (multiple myeloma), and 5 ALL-B (acute lymphoblastic leukemia) cases. Cases were considered JAM-C-positive if more than 20% of cells stained positive.

Figure 4. JAM-Cpos lymphoma B cells show a specific homing pattern to lymphoid organs, which can be influenced by anti-JAM-C antibodies. **A**, FACS analysis of B cells from blood of healthy donors, from patients with MZBL (JAM-Cpos), and from patients with CLL (JAM-Cneg) before injection into NOD/SCID mice. **B** and **C**, lymph nodes (LN) and bone marrow (BM), and spleen and peripheral blood (C) from NOD/SCID mice were obtained 1 hour after injection of human B cells and analyzed by FACS with anti-human CD19 and anti-human CD45 antibodies. Data indicate mean \pm SD of detected human B cells, per 10^6 cells acquired by FACS, per 10^6 injected B cells, either from healthy donors (black columns, $n = 8$), from patients with JAM-Cpos lymphoma (dark gray columns, $n = 5$), or from patients with JAM-Cneg lymphoma (light gray columns, $n = 3$). Differences in homing between different groups were analyzed using ANOVA, followed by Tukey *post hoc* analysis, $P < 0.05$. **D–F**, peripheral blood mononuclear cells from patients with JAM-Cpos lymphoma treated with control IgG or with anti-human JAM-C antibody were injected into NOD/SCID mice and bone marrow (**D**), spleen (**E**), and lymph nodes (**F**) were analyzed as in **B**. Columns show percentage of human B cells that homed to the organs relative to control (control = 100%). Data are mean \pm SD from 5 independent experiments; 2 mice per experiment. Differences in the homing between antibody-treated and control cells were analyzed using Student *t* test, $P < 0.05$.



(Fig. 4B and C). Interestingly, JAM-Cneg CLL B cells failed to reach lymph nodes and bone marrow but were found primarily in blood and spleen (Fig. 4B and C). Similarly to normal B lymphocytes, the homing of JAM-Cpos lymphoma B cells to bone marrow, spleen, and lymph nodes was substantially decreased by anti-JAM-C antibody (Fig. 4D–F).

Altogether, these results show that JAM-C on lymphoma B cells plays a crucial role in the homing to lymphoid organs, a function that can be partially blocked by anti-JAM-C antibodies.

Anti-JAM-C antibodies selectively block adhesion of JAM-C-positive B cells to mouse and human JAM-B

To determine the mechanism leading to the reduction of B-cell homing after anti-JAM-C treatment, we aimed to identify the molecular ligands of JAM-C on B cells. Although homotypic JAM-C/JAM-C and heterotypic JAM-C/JAM-B interactions have been reported, the endothelial ligand for JAM-C on B

cells has not yet been described. To investigate this interaction, adhesion assays were conducted. Culture plates were coated with recombinant human and murine JAM-B Fc, human JAM-C Fc, human VCAM-1 Fc as a positive, and JAM-A Fc as a negative control. The JAM-Cneg B-cell line Raji was transfected with full-length human JAM-C cDNA and mock-transfected cells were used as a control (Fig. 5A). Although mock-transfected Raji cells adhered only to VCAM-1, JAM-C-transfected Raji cells adhered to JAM-B and VCAM-1, but neither to JAM-C nor to JAM-A (Fig. 5B and C). Binding to VCAM-1 occurred through $\alpha 4$ integrin, as it was abrogated by incubation of cells with anti- $\alpha 4$ integrin antibody (data not shown). More importantly, incubation of JAM-Cpos Raji cells with anti-human JAM-C antibody selectively inhibited the binding of Raji cells to murine and human JAM-B without affecting the binding to VCAM-1 (Fig. 5B and D). In parallel experiments with K1718 (JAM-Cneg) and Jeko-1 (JAM-Cpos) B-cell lines, only Jeko-1

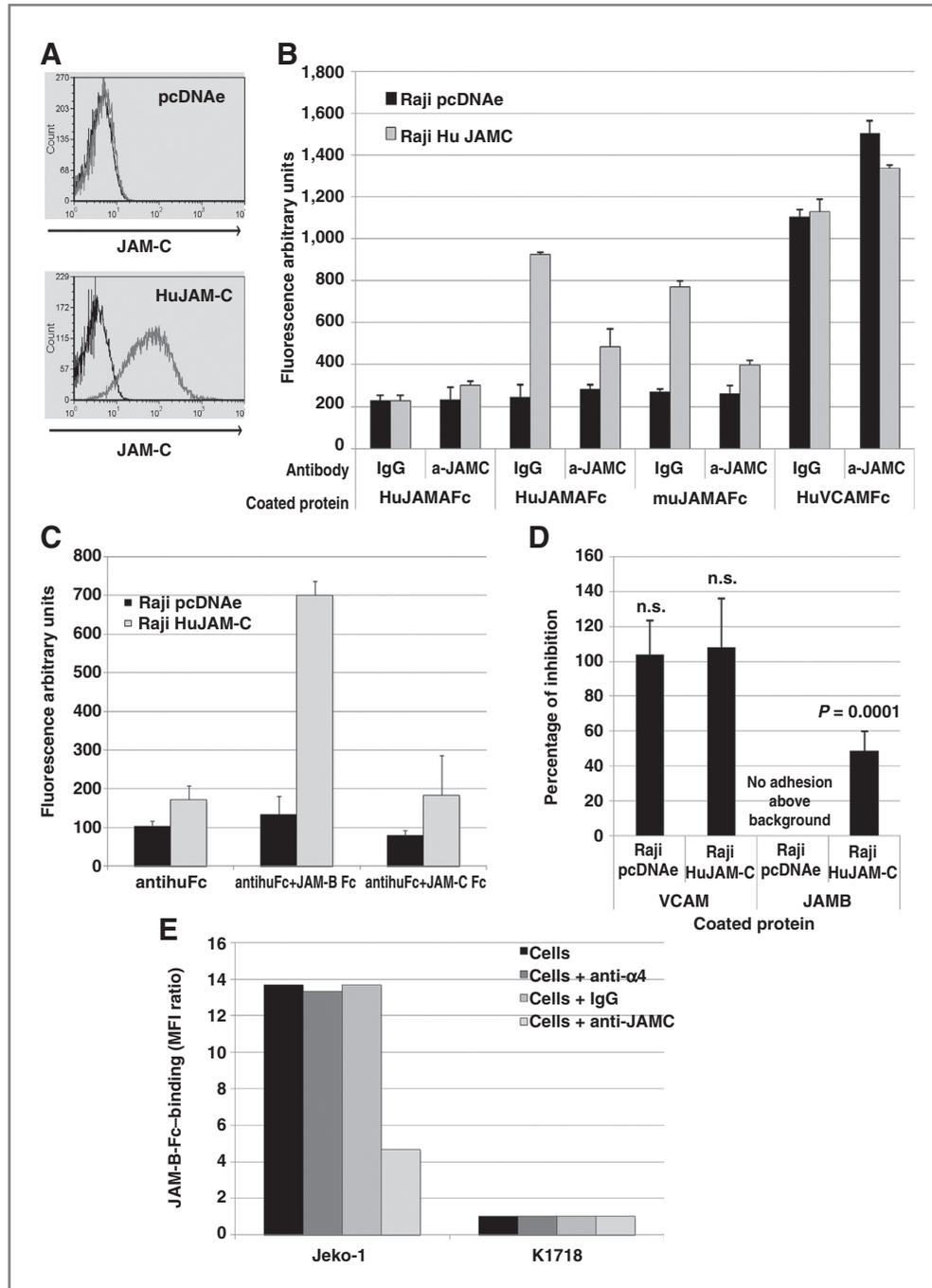


Figure 5. Human JAM-C interacts with JAM-B but not with JAM-C. **A**, surface JAM-C expression was analyzed in Raji cells by flow cytometry using anti-JAM-C antibodies. Raji cells were transfected with the pcDNA plasmid encoding neomycin resistance, without (pcDNAe), or with the full-length human JAM-C cDNA (HuJAM-C). **B**, Raji cells transfected with empty vector or with full-length human JAM-C were labeled with CFSE, incubated with rabbit IgG or with affinity-purified rabbit anti-JAM-C, and challenged for adhesion on coated soluble Fc-tagged molecules. Specific JAM-C-related adhesion occurred only on wells coated with JAM-B. Preincubation of the cells with anti-human JAM-C antibody selectively inhibited the binding of JAM-C-transfected cells to JAM-B and did not modify the binding to VCAM-1. The figure is representative of 6 independent experiments, 3 wells per condition. **C**, Raji cells transfected with empty vector or with full-length human JAM-C were labeled with CFSE and evaluated for adhesion on coated soluble Fc-tagged molecules. pcDNAe Raji cells did not adhere to any of the molecules tested, whereas HuJAM-C Raji cells adhered only to JAM-B. The figure is representative of 6 independent experiments, 3 wells per condition. **D**, anti-human JAM-C rabbit polyclonal antibody inhibits the binding of Raji cells to JAM-B but not to VCAM-1. Results show the percentage of cells incubated with rabbit anti-JAM-C related to the number of cells incubated with control rabbit IgG. Preincubation of cells with anti-human JAM-C selectively inhibited the binding of JAM-C-transfected cells to JAM-B. Differences in the percentage of inhibition between cells transfected with empty vector or with full-length human JAM-C were analyzed using Student *t* test, $P < 0.05$. **E**, anti-JAM-C antibodies, but not anti- α 4 integrin antibodies, inhibit the binding of JAM-B Fc to JAM-Cpos Jeko-1 cells. JAM-B binding was calculated as the ratio of JAM-B Fc MFI to Fc control MFI. The figure is representative of 3 independent experiments.

cells adhered to immobilized JAM-B Fc (Supplementary Fig. S6A).

To further document the interaction of JAM-B with JAM-C, binding assays of soluble JAM-B Fc were conducted with various B cell and non-B-cell lines, expressing different levels of JAM-C. A clear correlation between the level of expression of JAM-C and the binding of JAM-B was found (Supplementary Fig. S6B).

It has been previously described that JAM-B interacted also with $\alpha 4\beta 1$ integrin expressed by T cells and that this interaction occurred only on cells that concomitantly expressed JAM-C (21). To investigate whether this mechanism could be involved in B cells, Jeko-1 and K1718 cells (both $\alpha 4$ integrin-positive) were incubated with anti-JAM-C or anti- $\alpha 4$ integrin antibodies, and binding of soluble JAM-B Fc was analyzed by flow cytometry. As expected, JAM-B Fc bound to JAM-Cpos Jeko-1 cells but not to JAM-Cneg K1718 cells. Preincubation with anti- $\alpha 4$ integrin antibody did not affect this binding. However, incubation with anti-JAM-C antibody inhibited the binding of soluble JAM-B Fc by 66% (Fig. 5E). Similar results were obtained with JAM-Cpos-transfected Raji cells, in which preincubation with anti- $\alpha 4$ integrin antibody did not influence the binding of soluble JAM-B Fc (Supplementary Fig. S6C).

Taken together, these data identify JAM-B as the preferred cellular ligand for JAM-C expressed on B cells, and show that this binding, which occurs independently of $\alpha 4$ integrin, is specifically blocked by anti-JAM-C antibodies.

Surface plasmon resonance identifies JAM-B but not JAM-C as a ligand for JAM-C

To confirm the specificity of the JAM-C/JAM-B interaction at the molecular level, human soluble JAM-C-FLAG protein was covalently immobilized to a Biacore CM5 sensor chip. Immobilization was assessed by testing the binding of anti-JAM-C monoclonal and polyclonal antibodies (data not shown). Under these conditions, soluble recombinant JAM-B Fc and JAM-B FLAG bound to immobilized JAM-C, showing a clear JAM-B/JAM-C interaction, whereas binding of soluble JAM-C Fc to immobilized JAM-C was not detected (Fig. 6A). JAM-B FLAG was observed to dissociate rapidly, whereas the interaction of JAM-B Fc with JAM-C remained stable. This kinetic pattern may be due to the dimeric form of the Fc-tagged molecule forming a more stable interaction with JAM-C than the monomeric form of JAM-B FLAG. Homotypic JAM-C/JAM-C interaction was never observed under these conditions, even when injecting higher concentrations of JAM-C Fc. This suggests that JAM-C/JAM-B interaction is of much higher affinity than JAM-C/JAM-C binding. To validate these findings for our model of human cells injected into mice, we subsequently confirmed that human JAM-C binds to murine JAM-B to the same extent as to human JAM-B (Fig. 6B). Thus, our cell adhesion and Plasmon resonance data show that human and mouse JAM-B are ligands for JAM-C expressed by human B cells.

Vascular endothelial cells in lymphoid tissues express JAM-B and interact with JAM-C-positive B cells

JAM-B has been localized in cell-cell junctions of blood vessels, including HEVs in lymph nodes of normal mice (22). In

addition, the presence of JAM-B in murine bone marrow endothelial cells has been confirmed by flow cytometry and immunofluorescence (23). To ensure that JAM-B is also expressed by endothelial cells in NOD/SCID mice, lymph nodes and spleen sections were stained for JAM-B, and bone marrow was analyzed by flow cytometry for the presence of JAM-B-positive endothelial cells. As shown in Fig. 6C, JAM-B colocalized with PECAM-1 on blood vessels in spleen and lymph nodes, and CD45neg-PECAMpos bone marrow endothelial cells stained positive for JAM-B (Supplementary Fig. S7). To extend these results to humans, JAM-B staining on human lymphoid tissue was carried out. Colocalization of JAM-B and endothelial specific VE-cadherin (VE-CAD) was observed in the vasculature of human tonsils (Fig. 6C).

Finally, double staining experiments were carried out to determine the localization of JAM-Cpos B cells in relation to JAM-B-expressing endothelial cells of blood vessels. Lymph nodes of NOD/SCID mice, injected with JAM-Cpos human B cells, were collected 1 hour after injection and stained with anti-human CD20 and anti-mouse JAM-B antibodies. As shown in Fig. 6D, human B cells were found adjacent to JAM-Bpos endothelial cells.

Taken together, these data show that JAM-B is expressed by endothelial cells of blood vessels and that B cells interact with these cells, thus strongly suggesting that *in vivo* JAM-B/JAM-C interactions play a major role in B-cell homing to lymphoid organs.

Lymphoma engraftment is decreased by anti-JAM-C antibody treatment

To study whether the effect of anti-JAM-C antibodies on B-cell homing observed in short-term assays could be translated into a clinically relevant effect for lymphoma treatment, long-term assays were conducted. The JAM-Cpos B-cell line Jeko-1 (24) was injected into the tail vein of NOD/SCID mice. Animals were treated for 3 weeks with either anti-JAM-C antibodies or with control IgG (10 μ g/mouse, 2 times per week), and tumor burden was evaluated on day 24. The percentage of Jeko-1 cells detected in bone marrow, spleen, lymph nodes, and liver of anti-JAM-C-treated mice was significantly reduced as compared with control animals by 21%, 94%, 90%, and 66%, respectively (Fig. 7). No significant difference was found in the percentage of Jeko-1 cells detected in peripheral blood. These results show that anti-JAM-C antibody treatment not only decreased homing of B cells to lymphoid organs, but also long-term lymphoma engraftment.

Discussion

JAM-C is an adhesion molecule localized at endothelial tight junctions. In humans, it is also present in platelets, dendritic cells, and subsets of T, NK, and B cells (12). While the function of JAM-C at vascular tight junctions has been described (25–27), little is known about the role of JAM-C on circulating hematopoietic cells. Only few reports so far have suggested that the interaction of JAM-C on human lymphocytes with endothelial JAM-B might be involved in trafficking of these cells through endothelial barriers (14, 15). *In vivo* experiments with

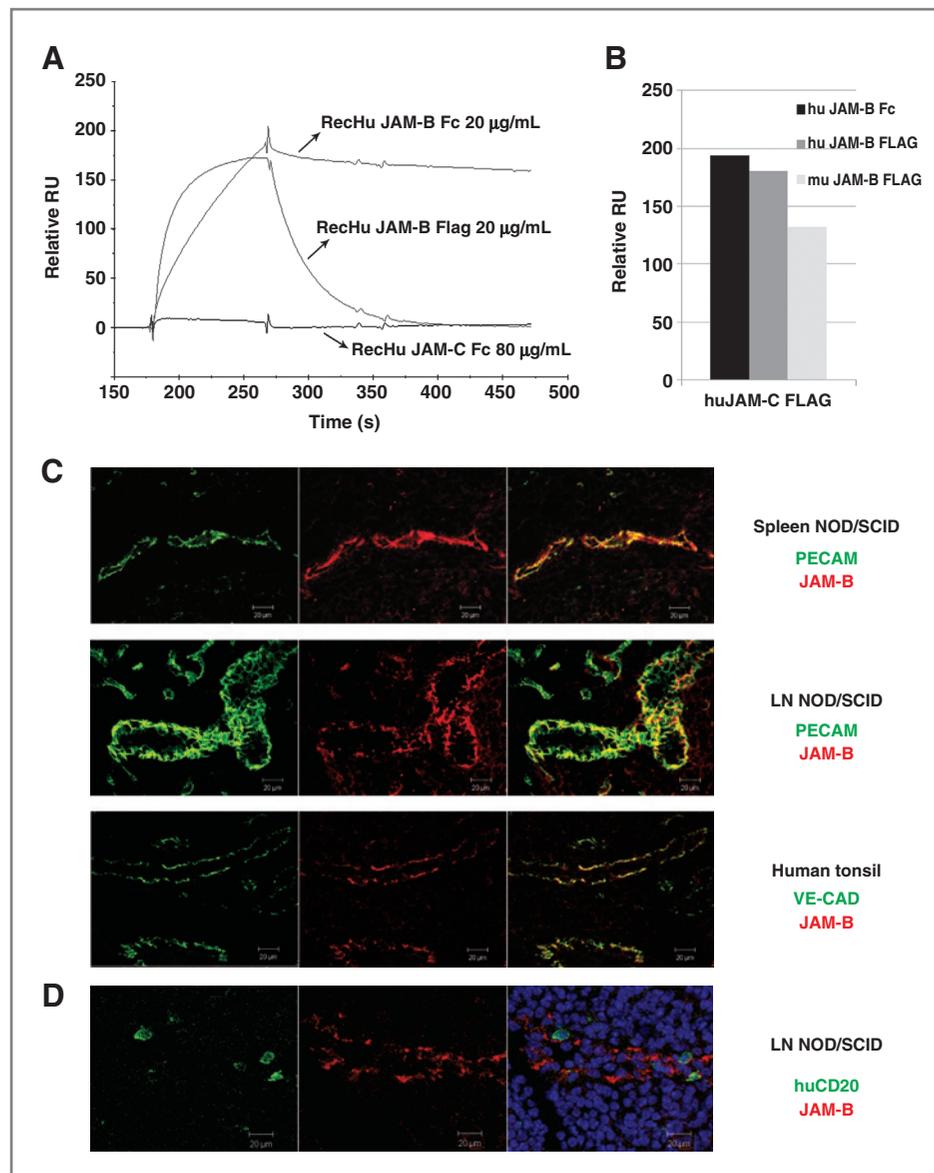


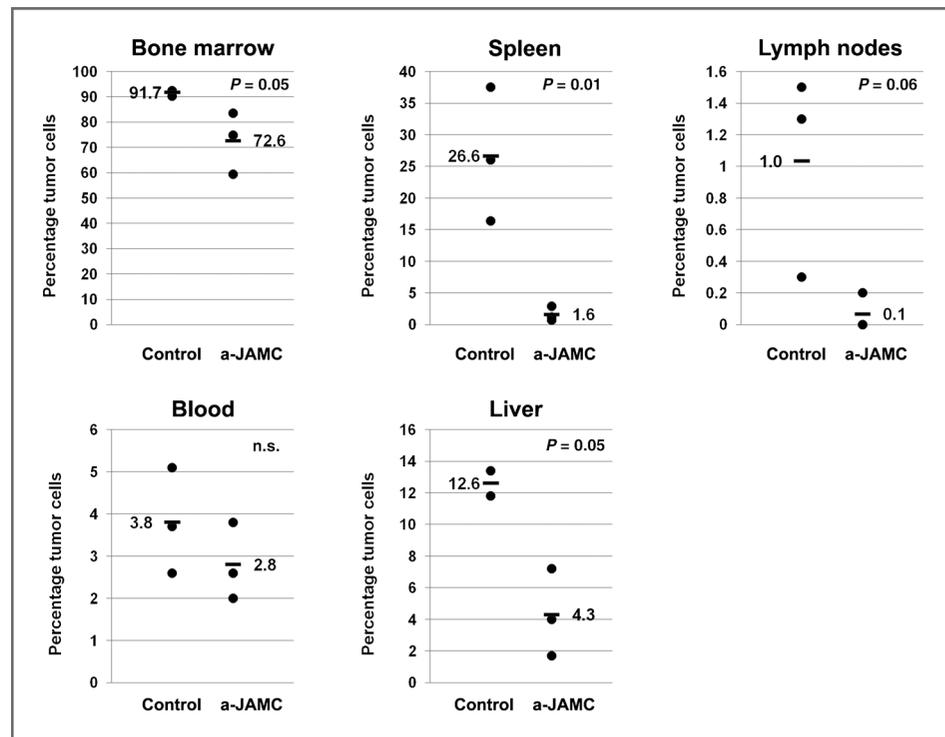
Figure 6. Analysis of JAM-B/JAM-C interactions with surface plasmon resonance and immunofluorescence. **A**, surface plasmon resonance analysis shows preferential JAM-B/JAM-C interaction. Sensograms of relative responses of soluble JAM-B Fc, JAM-B FLAG, and JAM-C Fc to immobilized JAM-C FLAG (the extracellular domain of the human soluble JAM-C FLAG protein was produced in BOSC cells and purified through an anti-FLAG affinity column). The background signal from a reference channel without soluble JAM-C was automatically subtracted. No association of JAM-C Fc to immobilized JAM-C was observed, whereas both JAM-B Fc and JAM-B FLAG associated with JAM-C. **B**, surface plasmon resonance analysis. Relative response units (resonance units, RU) were recorded at the end of injection and subtracted from RU at the beginning of injection to check for the comparative association ability of murine and human JAM-B molecules to immobilized human JAM-C FLAG. No major differences were observed between human or murine JAM-B FLAG protein. **C**, immunofluorescence of cryosections from spleen and lymph nodes (LN) of NOD/SCID mice and on cryosections from human tonsil. On mice tissues, rabbit polyclonal anti-JAM-B was localized on PECAM-expressing cells. On human tissues, rabbit polyclonal anti-JAM-B, produced by immunization of a rabbit with the soluble JAM-B FLAG molecule, was localized on VE-CAD^{pos} cells. **D**, immunofluorescence conducted on cryosections from lymph nodes of NOD/SCID mice obtained 1 hour after the inoculation of human B cells. JAM-C-expressing human B cells stained with huCD20 are localized adjacent to murine JAM-B^{pos} endothelial blood vessel cells.

B cells are further complicated by the fact that only human but not murine B cells express JAM-C.

Homing of circulating lymphocytes to secondary lymphoid organs is a multistep process, involving engagement of L-selectin, which mediates lymphocyte rolling along the luminal surface of HEVs, followed by activation of lymphocyte integrins

and transmigration through the endothelial cell layer. Once inside lymphatic tissues, B and T lymphocytes migrate toward specific microenvironments, such as B-cell follicles and the paracortex, respectively. As HEVs are absent in spleen, lymphocytes enter this organ via the terminal branches of the central arterioles, which guide them into the marginal zone (28,

Figure 7. Effect of anti-JAM-C treatment on long-term B-cell lymphoma engraftment. Jeko-1 cells (5×10^6 cells) were injected into the tail vein of NOD/SCID mice. Animals were treated with rabbit anti-JAM-C antibodies or with rabbit IgG during 3 weeks. At day 24, mice were sacrificed and bone marrow, spleen, lymph nodes, blood, and liver were analyzed by flow cytometry for the presence of Jeko-1 cells using anti-CD19 and anti-CD45 antibodies. Anti-JAM-C treatment reduced JAM-Cpos Jeko-1 lymphoma engraftment in bone marrow, spleen, lymph nodes, and liver. One representative experiment (3 mice per group) out of 2 is shown. Differences in the Jeko-1 percentages between antibody-treated and control mice were analyzed using Student *t* test.



29). In the bone marrow, migration of hematopoietic cells has been less well studied although it is known that selectins and integrins are involved in homing to this organ (30, 31) and that peripheral B lymphocytes enter through capillaries. Similar to normal B cells, the migration of malignant cells to specific lymphoid microenvironments constitutes a central aspect of B-cell lymphoma pathophysiology, and preventing lymphoma cells from reaching survival niches is important to stop tumor cell proliferation (2, 32).

In the present work, we show the critical importance of JAM-C in controlling the homing of normal and malignant human B cells to lymphoid organs. Using a xenogenic mouse model, we first described the involvement of JAM-C in the short-term homing of normal B cells to lymphoid organs and showed that anti-JAM-C antibodies impair the homing of normal cells to bone marrow, lymph nodes, and spleen. In addition, we showed that from a physiological mixture of JAM-C-positive and -negative B cells obtained from normal blood donors, the JAM-Cpos cells preferentially migrated to bone marrow, spleen, and lymph nodes. We next analyzed the migratory behavior of malignant B cells and showed that JAM-Cpos lymphoma B cells from MCL and MZBL homed in significantly lower numbers to lymph nodes compared with normal B lymphocytes, whereas no difference in homing to bone marrow and spleen was observed. The fact that lymphoma B cells do not behave exactly as normal B cells is not astonishing, as both cell types express a series of different adhesion molecules, in addition to JAM-C (33–36). The homing pattern of normal and JAM-Cpos lymphoma cells contrasted with the migration of JAM-Cneg cells from patients with CLL, which homed less efficiently to lymph nodes and bone marrow but accumulated

in spleen and blood. These results are in agreement with previous reports, in which it was also shown that CLL B cells failed to penetrate bone marrow and lymph nodes but homed instead preferentially to the spleen (3, 37). The reason why CLL B cells exhibit this migration pattern is not known. Because B-cell homing to spleen does not require integrin activation (38–40) it has been suggested that malignant cells fail to enter bone marrow or lymph nodes and rather use integrin-independent pathways to enter and accumulate in the spleen (3). We show here that JAM-C is not expressed by CLL B cells; whether other adhesion molecules might play a role remains to be defined.

The impaired homing of malignant B cells to bone marrow and lymph nodes in the NOD/SCID model seems to be in contradiction to the situation in humans. However, B-cell lymphomas typically take months to years to develop with B cells accumulating only slowly in lymphoid organs. Short-term homing assays certainly do not reflect this situation but help to understand the homing mechanisms of malignant cells. Taken together, our results show that splenic tissue may constitute the most supportive microenvironment for JAM-Cneg CLL cells, whereas JAM-Cpos cells from MCL and MZBL seek for specialized survival niches not only in the spleen but also in the bone marrow. Once inside the lymphoid organs, malignant cells survive and proliferate, thus contributing to disease progression, as shown by long-term experiments.

Treatment of malignant B cells with anti-JAM-C antibodies decreased their migration capacity to bone marrow, spleen, and lymph nodes. These experiments show that for JAM-Cpos B-cell lymphomas, JAM-C is an important player in the homing process. In accordance with the data of these short-term assays, our results also show that long-term administration of anti-

JAM-C antibodies significantly reduces the tumor burden in bone marrow, spleen, lymph nodes, and liver in mice injected with a JAM-Cpos B-cell line. Whether this reduction in lymphoma engraftment is due to the decreased entry of malignant B cells into supportive niches in the respective lymphoid organs, or to other mechanisms such as, for example, increased apoptosis or decreased proliferation, still needs to be elucidated.

It has been previously reported that B-cell homing to the spleen is integrin-independent, and that incubation of B cells with anti- $\alpha 4$ integrin antibodies reduces homing only to bone marrow and lymph nodes (3, 38–40). Our results now show that treatment with a combination of anti- $\alpha 4$ integrin and anti-JAM-C antibodies significantly reduces homing not only to bone marrow and lymph nodes, but also to the spleen. This might be clinically relevant, as engraftment of lymphoma cells to the spleen is an important issue in patients with B-cell lymphomas, in which the spleen constitutes a major reservoir for these tumors. Therefore, combination of different antibodies to reduce homing of B cells to several survival niches might be a promising new strategy for lymphoma therapy. In fact, migration antagonists are already proposed as part of combined therapies for B-cell lymphomas, together with chemotherapy and/or monoclonal antibodies such as rituximab (anti-CD20; ref. 2). In a mouse model of acute leukemia, AMD3100, a chemokine receptor antagonist for CXCR4, was shown to affect cell trafficking, to decrease tumor burden and to increase overall survival (41). Anti- $\alpha 4$ integrin and anti-CD44 antibodies are also currently being investigated in preclinical studies (4, 5, 7).

Several ligands for endothelial JAM-C have been described, including the integrin Mac-1 ($\alpha M\beta 2$, CD11c/CD18), JAM-B or JAM-C itself (reviewed in ref. 11). JAM-C has subsequently been defined as the leukocyte receptor for JAM-B expressed by endothelial cells (15, 42). The results of the surface plasmon resonance experiments are in agreement with these data as they showed selective and specific interaction of JAM-B with JAM-C. The interaction between both molecules was further shown by *in vitro* adhesion and binding assays with different B-cell lines, and by the specific blocking with anti-JAM-C antibodies. Unfortunately, these cell lines are not suitable for *in vivo* short-term homing experiments (data not shown).

Cunningham and colleagues showed that JAM-B could interact with $\alpha 4\beta 1$ integrin on T cells and that binding of JAM-B to JAM-C constituted a prerequisite for a subsequent

interaction of JAM-B with the integrin (21). Our results show that on B cells, the interaction of JAM-B occurs only between JAM-B and JAM-C, in a $\alpha 4\beta 1$ integrin-independent manner.

Comparable with C57BL/6 mice (22), we found expression of JAM-B on endothelial cells in lymph nodes, spleen, and bone marrow of NOD/SCID mice. Our results clearly show that JAM-C-expressing human B cells colocalize with these cells and that anti-JAM-C antibodies can block this interaction in an $\alpha 4$ integrin-independent way. We thus propose that in lymphoid organs vascular JAM-B constitutes the main ligand for JAM-C-expressing B cells.

In summary, we report a critical role for JAM-C in the homing of circulating B cells into lymphoid organs, as well as in B-cell lymphoma engraftment. Targeting JAM-C on malignant B cells could therefore constitute a novel approach in the treatment of JAM-Cpos B-cell lymphomas.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: C. Doñate, B.A. Imhof, T. Matthes

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Doñate, C. Ody, T. McKee, S. Ruault-Jungblut, N. Fischer, P. Ropraz

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Doñate, C. Ody, T. McKee, T. Matthes

Writing, review, and/or revision of the manuscript: C. Doñate, C. Ody, T. McKee, N. Fischer, B.A. Imhof, T. Matthes

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Doñate, C. Ody, B.A. Imhof, T. Matthes

Study supervision: C. Ody, B.A. Imhof, T. Matthes

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Homing of Human B Cells to Lymphoid Organs and B-Cell Lymphoma Engraftment Are Controlled by Cell Adhesion Molecule JAM-C

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