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Anti-JAM-C therapy eliminates tumor engraftment in a xenograft model of mantle cell lymphoma

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ABSTRACT

Junctional adhesion molecule (JAM)-C is a member of the JAM family, expressed by a variety of different cell types, including human B lymphocytes and some B-cell lymphoma subtypes—in particular, mantle cell lymphoma (MCL). Treatment with anti-JAM-C pAbs reduces homing of human B cells to lymphoid organs in a NOD/SCID mouse model. In the present study, the role of JAM-C in the engraftment of human lymphoma B cells in mice was investigated. Administration of novel anti-JAM-C mAbs reduced tumor growth of JAM-C$^+$ MCL cells in bone marrow, spleen, liver, and lymph nodes of mice. Treatment with anti-JAM-C antibodies significantly reduced the proliferation of JAM-C-expressing lymphoma B cells. Moreover, the binding of anti-JAM-C antibodies inhibited the phosphorylation of ERK1/2, without affecting other signaling pathways. The results identify for the first time the intracellular MAPK cascade as the JAM-C-driven signaling pathway in JAM-C$^+$ B cells. Targeting JAM-C could constitute a new therapeutic strategy reducing lymphoma B-cell proliferation and their capacity to reach supportive lymphoid microenvironments. *J. Leukoc. Biol.* 100: 843–853; 2016.

Introduction

MCL constitutes 3–10% of all malignant non-Hodgkin B-cell lymphomas and is characterized by the chromosomal translocation t(11;14)(q13;q32), which results in overexpression of cyclin D1 and cell cycle deregulation [1]. The median survival of patients remains at only 4–5 yr, despite development of new drugs and improvement in therapeutic strategies. Most patients develop refractory MCL that is resistant to current medications with a median survival of less than 2 yr [2, 3].

JAM-C is a member of the junctional adhesion molecule family, with 2 extracellular Ig-like domains and a cytoplasmic tail with a PDZ binding motif [4]. It has been described as an endothelial adhesion molecule localized in tight junctions of vessels but is also expressed by human B cells, platelets, activated T cells, and NK cells [5]. Our group has shown that JAM-C is present on the surface of malignant lymphoma B cells and that its differential expression allows classification into 2 types of B-cell malignancies: JAM-C$^-$ (CLL, follicular lymphoma, and diffuse large B-cell lymphoma) and JAM-C$^+$ (MCL, marginal zone B-cell lymphoma, and hairy cell leukemia) lymphomas [6, 7]. We have also demonstrated in a NOD/SCID mouse model that short-term treatment with anti-JAM-C pAbs blocks the adhesion of JAM-C–expressing B cells to their ligand JAM-B and therefore reduces cell migration to bone marrow, lymph nodes, and spleen [6].

Recent work has demonstrated that tight junction molecules are crucial components of signaling pathways, which regulate cell polarity and vascular permeability, as well as cell migration, proliferation, and differentiation [8, 9]. In the current study, we examined the effect of several newly developed anti-JAM-C mAbs on the homing of lymphoma B cells to lymphoid organs and on the in vivo growth of JAM-C$^+$ MCLs. Subsequently, we investigated the effect of anti-JAM-C antibody on B-cell proliferation and used phosphospecific antibodies and flow cytometry to monitor basal and induced signaling molecules on primary JAM-C$^+$ lymphoma B cells, as well as the effect of anti-JAM-C antibody binding on these pathways. Our results showed that administration of anti-JAM-C mAbs drastically reduced the development of MCLs in the NOD/SCID mouse model. Furthermore, ligation of anti-JAM-C antibodies decreased proliferation of malignant B cells. Among the different signaling molecules studied, we found a specific decrease in ERK1/2 phosphorylation after anti-JAM-C binding, pointing to the

Abbreviations: CLL = chronic lymphocytic leukemia, EdU = 5-ethynyl-2′-deoxyuridine, hJAM = human JAM, JAM = junctional adhesion molecule, MCL = mantle cell lymphoma, MPL = monophosphoryl-lipid A, NOD/SCID = NOD.CB17-Prkdcscid/J, pAb = polyclonal antibody, TDM = trehalose dicorynomycolate

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intracellular MAPK cascade as the JAM-C driven signaling pathway in JAM-C$^+$ B-cell tumors. These results support JAM-C as a potential new target for the treatment of JAM-C$^+$ B-cell lymphomas and, in particular, MCL.

MATERIALS AND METHODS

Human samples and cell lines

After informed consent was obtained, peripheral blood samples were taken from healthy blood donors from the local blood transfusion center and from patients with leukemic B-cell lymphoma from the Hematology Service of the Geneva University Hospital. They were used according to procedures approved by the local ethics and human experimentation committees and the Declaration of Helsinki. Mononuclear cells were collected after standard separation on Ficoll-Paque (GE Healthcare, Pittsburgh, PA, USA). B cells were enriched by negative selection with a human B-cell enrichment kit (EasySep; StemCell technologies, Vancouver, BC, Canada), according to the manufacturer's instructions. We routinely obtained a purity of >90% (data not shown). The cells were used immediately for the experiments. The human cell line Jeko-1 was a kind gift from J. A. Martinez-Climent (Laboratory of Molecular Oncology, Center for Applied Medical Research, University of Navarra, Spain). The cell lines Raji and MDCK were purchased from the American Type Culture Collection (Manassas, VA, USA). The phenotype of the cell lines was tested and authenticated by flow cytometry before use.

Production of anti-JAM-C mAbs

Two rats received 3 injections of 50 μg (s.c.) affinity-purified soluble JAM-C flag: 1 injection every 10 d. One injection contained Ribi MPL+TDM+cell wall skeleton adjuvant (MPL+TDM cell wall skeleton; Sigma-Aldrich, Bruchs, Switzerland) and 2 injections contained MPL+TDM. Fusion of inguinal lymph node cells with Sp2/0 myeloma cells was performed 2 d after the last injection. Cells were cultured in Hybridoma Fusion and Cloning Supplement (Roche, Basel, Switzerland) and 10% Hybmix (Sigma-Aldrich). Supernatants were tested 11 d after the fusion by flow cytometry on MDCK-wt, MDCK transfected with human JAM-C (MDCK-hJAM-C) and MDCK transfected with murine JAM-C (MDCK-mJAM-C). Antibodies positive for MDCK-hJAM-C and negative for MDCK-wt and MDCK-mJAM-C were selected as human-specific anti-JAM-C mAbs.

B-cell cultures

Human B lymphocytes were seeded at 5×10^5/well in 24-well plates in RPMI 1640 culture medium containing L-glutamine and supplemented with 10% FCS, 10 mM HEPES (pH 7.4), and antibiotics (Thermo Fisher Scientific, Waltham, MA, USA). The cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2.

Surface plasmon resonance assays

Surface plasmon resonance assays were performed as has been described previously [6]. In brief, 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; GE Healthcare). Background signals were automatically subtracted. Antibodies (30 μl) were injected (Kinject procedure; GE Healthcare) in running buffer (400 μM Tris–HCl, pH 7.4, containing 145 mM NaCl) at a flow rate of 20 μl/min.

Flow cytometry and proliferation assays

Cell surface expression of JAM-C was assessed by flow cytometry with anti-JAM-C pAbs and mAbs [6, 7]. For proliferation assays, cells cultured on complete RPMI 1640 medium plus cytokines (25 ng/ml IL-2, 50 ng/ml IL-4, 25 ng/ml IL-10; PeproTech, Rocky Hill, NJ, USA) and human CD40L (800 ng/ml; PeproTech) were treated with either 10 μg/ml of the anti-JAM-C mAbs H225 and Hj20 or with 4.5 μm diameter epoxy-activated magnetic beads (Thermo Fisher Scientific) coated overnight with anti-JAM-C antibodies (or anti-CD45 for positive controls), according to the manufacturer's instructions. Rat IgG1 isotype and nonactivated cells were analyzed as negative controls. Cell proliferation was assessed by 2 different methods: DRAQ5 (Biostatus) and EdU (Thermo Fisher Scientific Life Sciences). At different time points, the cells were stained with DRAQ5 for 25 min before analysis, and the percentage of B cells in S and G_2/M phases was measured by flow cytometry. Alternatively, EdU was added to the culture medium at 10 μM (final concentration), and the cells were incubated for 16 h. Samples were then harvested, and proliferating EdU$^+$ cells were detected by flow cytometry. In some experiments the MEK1/2 inhibitor U0126 (Cell Signaling Technology, Danvers, MA, USA) was used at a concentration of 10 μM.

Chemokine receptors and cell surface adhesion molecules were assessed [6]. For cyclin D1 staining, directly conjugated anti-cyclin D1-FITC (Abcam, Cambridge, MA, USA) was used.

All cytometry experiments were performed on a Navios flow cytometer (Beckman Coulter, Brea, CA, USA) using FCS Express software (De Novo Software, Glendale, CA, USA).

Intracellular phosphospecific flow cytometry

Treated and untreated cells were fixed by adding 37% formaldehyde (Sigma-Aldrich) directly into the culture medium to obtain a final concentration of 2% formaldehyde. The cells were incubated for 15 min at RT, permeabilized by resuspending in ice-cold MeOH, incubated at 4°C for at least 10 min, and then stored at −20°C. For staining, the cells were washed twice in staining medium (PBS containing 0.1% BSA) and resuspended at 0.1–0.5 × 10^6 cells/50 μl. Optimal concentrations of the phosphospecific mAbs ERK1/2 (pT202/pY204) Alexa-488, Akt (pS473) Alexa-488, p38 MAPK (pT180/pY182) Alexa-488, Stat3 (pY705) Alexa-488, and JNK (pT183/pY185) PE (all from BD Biosciences, San Jose, CA) were added to the cell suspensions and incubated for 30 min at RT. The samples were washed with staining medium, pelleted, resuspended, and analyzed by flow cytometry. Inhibition of Erk phosphorylation was confirmed with the inhibitor U0126 at the same concentration as above.

Chemotaxis assays

Jeko-1 cells (5×10^5) were incubated in complete RPMI 1640 medium with either control IgG or anti-JAM-C mAb for 30 min at 37°C in 5% CO_2. The cells were resuspended in 200 μl and added to the top chamber of a Transwell culture insert (Costar; Corning, Inc., Corning, NY, USA) with a diameter of 6.5 mm and a pore size of 5 μm. Filters were then transferred to wells containing complete medium, with or without 150 ng/ml CXCL12 (PeproTech). At 1.5 h later, the cells were harvested from the lower well and counted by flow cytometry.

Confocal microscopy

Jeko-1 cells were incubated with H225 mAb or control IgG at 37°C for 3 and 24 h and mounted on poly-L-lysine-pretreated slides. The cells were fixed with 2% PFA, washed with PBS and permeabilized with 0.1% Triton X-100. The samples were then washed with 0.1% BSA-PBS and incubated for 1 h with phalloidin-FITC. After 3 washes, the nuclei were counterstained with DAPI (200 ng/ml; Thermo Fisher Scientific Life Sciences), and the slides were mounted in Moewiol (Sigma-Aldrich). Fluorescence was analyzed with an LSM 510Meta confocal microscope (Zeiss, Oberkochen, Germany).

Mice

NOD/SCID mice were obtained from Charles River Laboratories (Burlington, MA, USA) and bred under defined flora conditions at the animal facility of the University Medical Center, University of Geneva, in sterile microisolator 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 were incubated 30 min with mAb anti-JAM-C or with control isotype (10 μg/ml) and injected into the tail vein of nonirradiated 5–8-wk-old NOD/SCID mice (4–12 × 10^6 cells per

mouse). One hour after injection, mice were killed and bone marrow, spleen, liver, and lymph nodes (mesenteric, inguinal, brachial, axillary, and cervical) were collected. Human B cells from the different organs were detected by flow cytometry with 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 as described previously [6].

Lymphoma development assays in NOD/SCID mice

Jeko-1 cells (5×10^6 cells) were injected intravenously into 5–8 wk-old NOD/SCID mice. Six or 10 d after the injection, the mice were randomly distributed and treated with either rat IgG1 (control group) or anti-JAM-C antibodies (50 μg/mouse). Subsequently, the antibodies were administrated intravenously twice a week. The mice were monitored for general condition and weight loss and killed at day 22 or 26. Bone marrow, spleen, liver, and lymph nodes were collected and analyzed for the presence of Jeko-1 cells by flow cytometry with human-specific anti-CD45 and anti-CD19 antibodies.

Statistical analysis

Prism 6 software was used for statistical analysis (GraphPad, San Diego, CA, USA). The significance of differences between groups was determined with Student's *t* test or ANOVA followed by Dunnett's post hoc test. Results are expressed as means ± SEM, with $P < 0.05$ indicating significance.

RESULTS

In a previous study, anti-JAM-C pAbs blocked the adhesion of JAM-C-expressing B cells to their ligand JAM-B, thus inhibiting the homing of malignant JAM-C$^+$ B lymphocytes to bone marrow, spleen, and lymph nodes in NOD/SCID mice.

Generation of anti-JAM-C mAbs

To determine whether JAM-C could constitute a new therapeutic target for the treatment of B-cell lymphomas, we immunized rats with recombinant human JAM-C protein. We then applied classic hybridoma technology to generate specific mAbs. We selected 6 antibodies for further characterization (2 IgG1 and 4 IgG2a isotype), based on specific recognition of the human JAM-C molecule, which was confirmed by flow cytometry and surface plasmon resonance assays (**Fig. 1A, B**).

Anti-JAM-C mAbs decrease the homing of JAM-C$^+$ primary B cells to lymphoid organs

To elucidate whether the new anti-JAM-C mAbs and the previously described pAbs have similar blocking effects on the migration of B cells [6], we performed short-term homing assays. Human B cells from peripheral blood were incubated with the anti-JAM-C mAbs or with control IgG and then injected into NOD/SCID mice. One hour later, the mice were killed, and the human B cells from bone marrow, spleen, liver, and lymph nodes were quantified by flow cytometry, using human-specific anti-CD45 and anti-CD19 antibodies. The anti-JAM-C antibody H225 (IgG1) efficiently reduced the homing of B cells to the 4 organs, whereas Hj20 (IgG1) and 3 other novel IgG2a antibodies did not influence the trafficking of the cells (Fig. 1C and data not shown). Similar to H225, the antibody H36.6 (IgG2a) also decreased the homing of the cells to the lymphoid organs but with reduced efficiency. H225 was therefore chosen for all subsequent experiments. Flow cytometry competition assays between H225 and Hj20 showed noncompetitive binding, indicating that the epitopes recognized by the 2 antibodies are different (data not shown).

Development of lymphoma is reduced by anti-JAM-C antibody treatment

We next investigated whether the H225 antibody also influences the development of B-cell lymphomas. We first examined the tumor growth pattern of the MCL cell line Jeko-1 in NOD/SCID mice. As expected, intravenous injection of Jeko-1 cells led to progressive infiltration of all the organs studied (Supplemental Fig. 1) and mice had to be killed at d 26 because of a decline in health status.

To demonstrate the therapeutic potential of anti-JAM-C antibodies, we treated the mice with repeated intravenous injections of H225 or control IgG, starting 6 days after the injection of Jeko-1 cells. The percentages of tumor cells were then quantified by flow cytometry on d 26. Mice treated with H225 showed drastically reduced quantities of lymphoma cells in spleen, liver, bone marrow, and lymph nodes, with a decrease of 100%, 99%, 95%, and 100%, respectively, compared with control mice. This inhibition correlated with a clearly visible reduction in the sizes of spleen and lymph nodes (**Fig. 2**).

As treatment for lymphoma in the clinics is normally applied to patients with an already well-established disease, we then studied the effects of anti-JAM-C treatment, starting with intravenous injections of H225 only 10 d after injection of Jeko-1 cells. The percentage of tumor cells was again quantified by flow cytometry on d 22. With this treatment schedule, engrafted tumor cells in bone marrow and lymph nodes were not decreased by H225 treatment and remained unchanged compared with the number in control mice, whereas in spleen and liver, the number of tumor cells was decreased by 99% and 83%, respectively, comparable to mice that had been treated with H225 from d 6 onward (**Fig. 3**).

To explain these results, we hypothesized that the H225 antibody, in addition to its inhibitory effects on the homing of recirculating B cells, also affects tumor growth and engraftment by other mechanisms. Depending on the infiltrated organ, the corresponding microenvironment may influence differentially the protection of tumor cells from these effects.

Anti-JAM-C antibodies do not influence activation and expression of cell surface adhesion molecules, actin polymerization, or chemotaxis of lymphoma B cells

To investigate whether the H225 antibody, in addition to its effect on B-cell migration, influences cell activation or the expression of other adhesion molecules and chemokine receptors involved in homing, we performed in vitro experiments. Jeko-1 cells were cultured and incubated with H225 antibody or isotype control for 3, 24, 48, or 72 h and then analyzed by flow cytometry for the expression of CD19 and BCL6; the chemokine receptors CCR6, CCR4, CCR7, and CXCR4; the adhesion molecules α-4 integrin, β-2 integrin, platelet endothelial cell adhesion molecule, and CD44; and the B-cell activation markers CD38 and CD69. No differences in expression were detected for any of these markers (Supplemental Fig. 2 and data not shown). The expression of the molecules was also analyzed in vivo on Jeko-1 cells recovered from the bone marrow of NOD/SCID

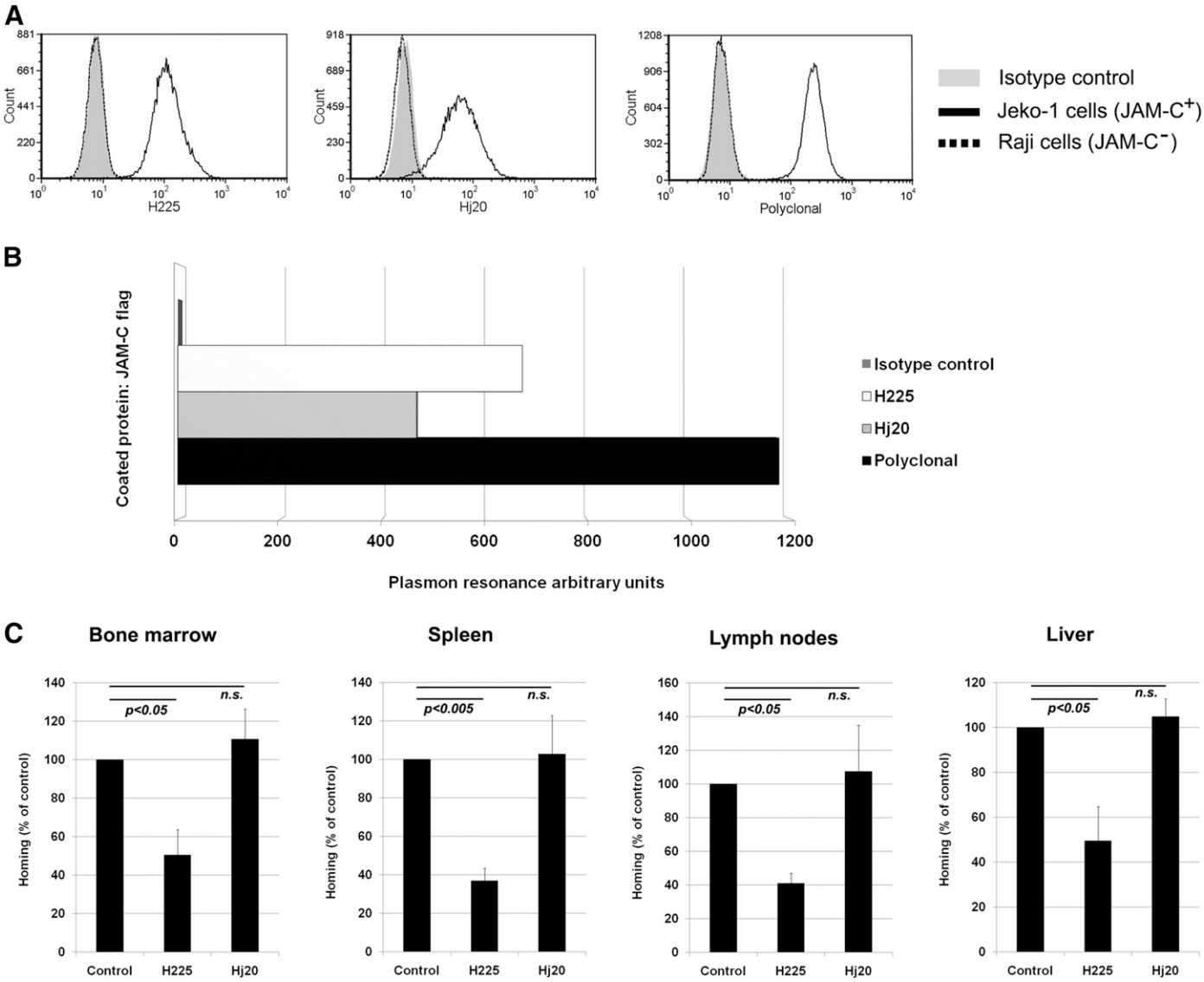

Figure 1. Characterization of anti-JAM-C mAbs. The anti-JAM-C antibody H225 reduces the homing of B cells to lymphoid organs. (A) Surface JAM-C expression was analyzed on Jeko-1 cells (JAM-C$^+$) and Raji cells (JAM-C$^-$) by flow cytometry with 6 anti-JAM-C mAbs. Histograms of H225 and Hj20 (IgG1) are shown as an example. Staining with the pAb was the positive control. (B) Surface plasmon resonance analysis of soluble isotype control, anti-JAM-C mAbs H225 and Hj20, and pAbs to immobilized JAM-C FLAG. Plasmon resonance arbitrary units were recorded at the end of the injection and subtracted from arbitrary units at the beginning of the injection. The background signal from a reference channel without soluble JAM-C FLAG was automatically subtracted. (C) Human B cells treated with control IgG or with the anti-JAM-C antibody H225 or Hj20 were injected into NOD/SCID mice. One hour later, bone marrow, spleen, lymph nodes, and liver were collected, and B cells were detected and quantified by flow cytometry with anti-CD19 and -CD45 antibodies. The effect of the antibodies is expressed relative to isotype control (100%). Data are means ± SEM of results in 4 experiments. The differences between groups were determined by ANOVA, followed by Dunnett's post hoc analysis.

mice treated with H225 or isotype control (Fig. 3). Again, no differences in any of the markers were observed (**Fig. 4A**).

To evaluate whether H225 directly influences the chemotactic behavior of Jeko-1 cells, we performed in vitro Transwell assays. Jeko-1 cells express CXCR4 (Supplemental Fig. 2) and migrate toward the chemokine CXCL12. Preincubation of the cells with H225 did not alter their chemotactic response to CXCL12 (Fig. 4B).

Cell migration and homing largely depends on cytoskeleton activities. To examine the ability of the anti-JAM-C antibody H225 to induce changes in the cytoskeleton, we studied the intracellular filamentous actin scaffold (F-actin). Jeko-1 cells were treated with 10 μg/ml H225 or control IgG. As shown in Fig. 4C, incubation with the antibody did not modify the polymerization of actin, visualized by fluorescent phalloidin staining. Intracellular F-actin content was also quantified by FACS. No differences were detected between H225-treated and control cells (data not shown). These results demonstrate that the mAb H225 does not induce actin cytoskeleton polymerization or rearrangement. Taken together, these results indicate that the anti-JAM-C mAb H225 does not affect any of the tested mechanisms.

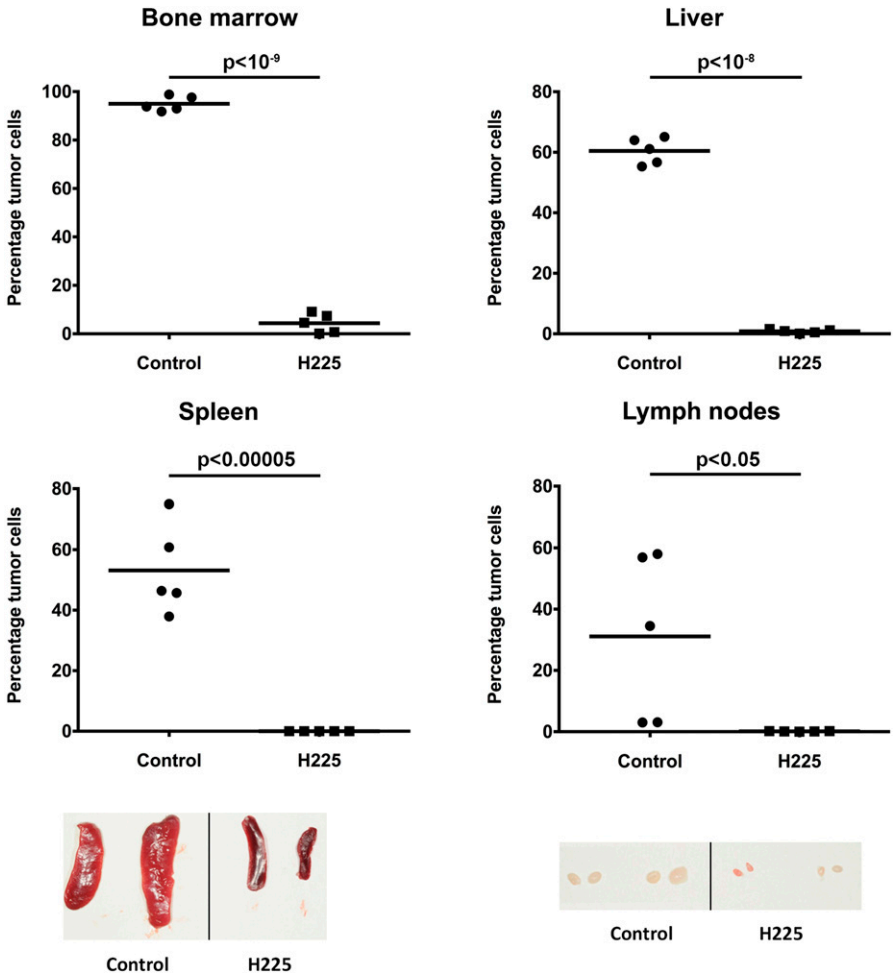

Figure 2. Treatment with anti-JAM-C antibody H225 eradicates MCL. Jeko-1 cells (5×10^6 cells) were injected into NOD/SCID mice. Animals were treated with either rat IgG1 (control group) or H225 antibodies, 50 μg/mouse, beginning 6 days after inoculation of the cells. At d 26, mice were killed, and Jeko-1 cells were detected by flow cytometry with anti-CD19 and -CD45 antibodies. One representative experiment of 2 is shown ($n = 5$). Differences in the percentage of Jeko-1 cells between mice treated with H225 antibody and control mice were analyzed with Student's *t* test.

Anti-JAM-C antibodies affect proliferation of human lymphoma B cells

The inhibition of lymphoma homing mediated by H225 antibodies (Fig. 1) cannot explain the total eradication of tumors in the different organs (Fig. 2). Additional antibody effects on cell proliferation are a possibility. Along this line, we first examined the effect of anti-JAM-C antibodies on proliferation of JAM-C$^+$ B cells by using H225 or Hj20 anti-JAM-C antibodies linked to epoxy magnetic polystyrene beads. These beads allow optimal presentation of the antibodies to B cells in culture, mimicking in vivo interactions with other cells [10]. The cell cycle was evaluated after 3 h by DRAQ5 staining. H225 coupled to beads decreased the proliferation rate of Jeko-1 cells by 15% at 3 h (**Fig. 5A**). Hj20 antibody, used as the control, had no effect. Similar results were obtained at 5 h (data not shown). This inhibition of proliferation was not caused by a decrease in cell viability or by an induction of apoptosis, given that no differences in 7AAD or annexin-V staining were observed (data not shown).

Cell lines are highly proliferating cells reflecting adaptation and selection related to long-term in vitro culture conditions. Therefore, we next examined the ability of H225 antibody to inhibit the proliferation of primary lymphoma B cells. JAM-C$^+$ B cells from patients with leukemic MCL were cultured in the presence of recombinant CD40L and cytokines and either H225 or Hj20 antibody or isotype control. Cell proliferation was evaluated at d 3 of culture by flow cytometry with DRAQ5 or EdU staining. A significant inhibition in cell proliferation was found when cells were incubated with H225 anti-JAM-C antibody (33.0 ± 3.9% and 29.9 ± 3.5%, as measured by EdU and DRAQ5, respectively; Fig. 5B), but not with Hj20.

To test whether the effect on cell proliferation observed in vitro would explain our in vivo results, we analyzed cell proliferation of human lymphoma cells recovered from mice. For this experiment, mice were first injected with Jeko-1 cells and then treated with H225 antibody, as described above. Several days later, the mice were injected intravenously with EdU and killed the next day, and proliferation was measured in cells collected from bone marrow. Lymphoma cells from H225-treated mice showed a clear 35% decrease in proliferation compared with those in the controls (Fig. 5C).

Because MCL cells, including Jeko-1, overexpress the cell cycle protein cyclin D1, we investigated a potential direct effect of H225 antibody on its expression. After validation of the method of quantification of cyclin D1 by flow cytometry (Supplemental Fig. 3A), Jeko-1 cells were incubated with H225 antibody or control isotype for different periods. No differences were

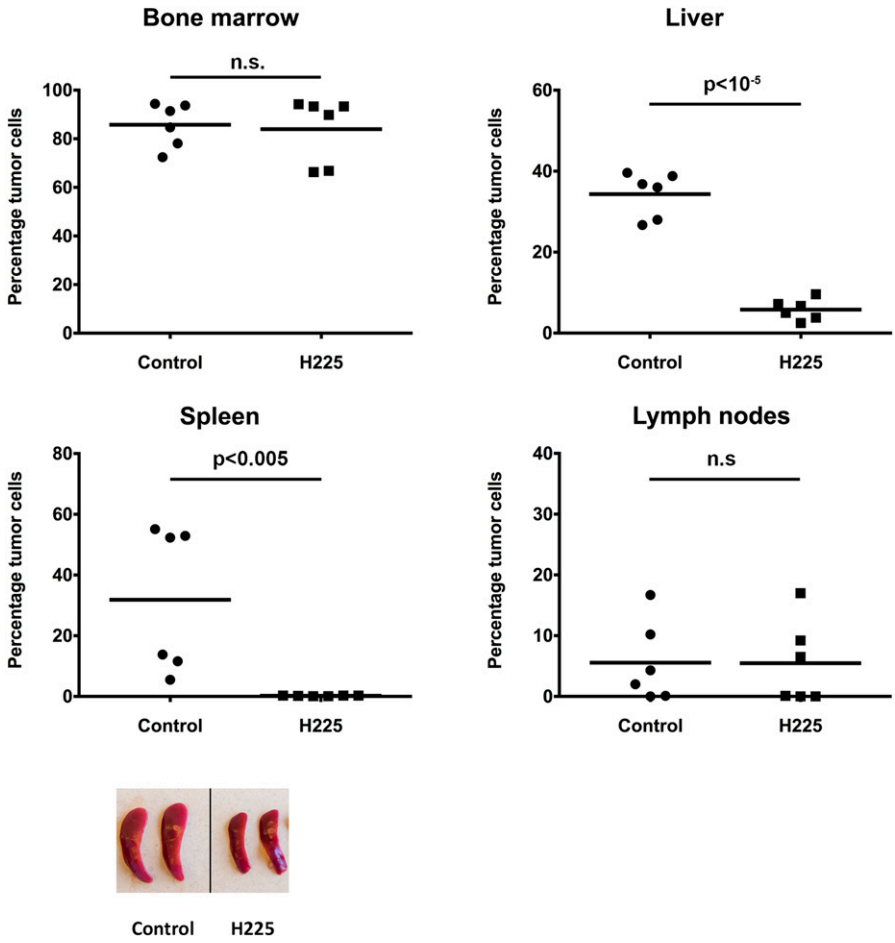

Figure 3. Late treatment with the anti-JAM-C antibody H225 reduces MCL engraftment only in liver and spleen. Jeko-1 cells (5×10^6 cells) were injected into NOD/SCID mice. The animals were treated with either rat IgG1 (control group) or H225 antibodies (50 μg/mouse) beginning 10 days after inoculation of the cells. At day 22, mice were killed, and Jeko-1 cells were detected by flow cytometry with anti-CD19 and -CD45 antibodies. Pooled data from 2 independent experiments are shown ($n = 6$). Differences in the percentage of Jeko-1 cells between mice treated with H225 antibody and control mice were analyzed by Student's t test.

observed in the percentage of cyclin D1$^+$ cells in H225-treated and control cells (Supplemental Fig. 3B). Similar results were obtained when primary MCL cells were incubated with H225: no effect on cycle D1 expression was observed (data not shown).

Altogether, these results demonstrate that anti-JAM-C H225 antibodies exert a direct inhibitory effect on the proliferation of the MCL Jeko-1 cell line and primary lymphoma cells, without having a direct effect on the expression of cyclin D1.

The binding of anti-JAM-C mAbs decreases the phosphorylation of ERK1/2

To investigate the mechanism behind the H225-triggered decrease in cell proliferation, we monitored phosphorylation profiles of known B-cell intracellular pathways at single cell levels by phosphospecific flow cytometry. First, signaling pathways were analyzed in Jeko-1 cells by using phosphospecific antibodies for p38, ERK1/2, JNK (MAPKs), Stat3, and Akt (the PI3K/Akt/mTOR cell survival pathway). Unstimulated Jeko-1 cells exhibited high basal (constitutive) phosphorylation levels of ERK1/2, JNK, and Akt, whereas phosphorylation of p38 and Stat3 was not detected (**Fig. 6A**). Jeko-1 cells were then incubated with either the anti-JAM-C mAb H225 or with isotype control for 30 min or 3 h. The binding of anti-JAM-C antibodies led to a decrease in ERK1/2 phosphorylation by 25% or 34%, respectively, without affecting the phosphorylation of the other signaling proteins JNK and Akt (Fig. 6B). The phosphorylation of p38 and Stat3 remained undetectable. Addition of the Erk inhibitor U0126 to cultures completely abrogated ERK1/2 phosphorylation and Jeko-1 cell proliferation (Supplemental Fig. 4).

To evaluate whether H225 antibody also influences the phosphorylation profiles of primary lymphoma cells, we investigated 10 patients with JAM-C$^+$ leukemic B cells. Basal phosphorylation levels were low for all 5 phosphoproteins studied (data not shown). Lymphoma B cells were then activated with CD40L and cytokines for 15 min and incubated with either H225 antibody or with isotype control. The 15 min period was selected based on a kinetic study performed on primary normal B cells, which produced a robust but not maximum phosphorylation of the 5 signaling proteins evaluated (data not shown). In accordance with previously published results [11–13], phosphoprotein expression patterns were observed to vary widely among the cases. Phosphorylation of ERK1/2 was detected in all patients after CD40L activation and was decreased in 8 of 10 samples incubated with H225 (Fig. 6C), similar to the effect on Jeko-1 cells. The effect of the antibody relative to isotype control (100%) was a mean reduction of 15.3% in the phosphorylation of ERK1/2 ($P < 0.005$). On the other phosphoproteins studied, no differences were observed upon H225 treatment; in some samples, CD40L activation did not lead to phosphorylation of some of the 5 phosphoproteins.

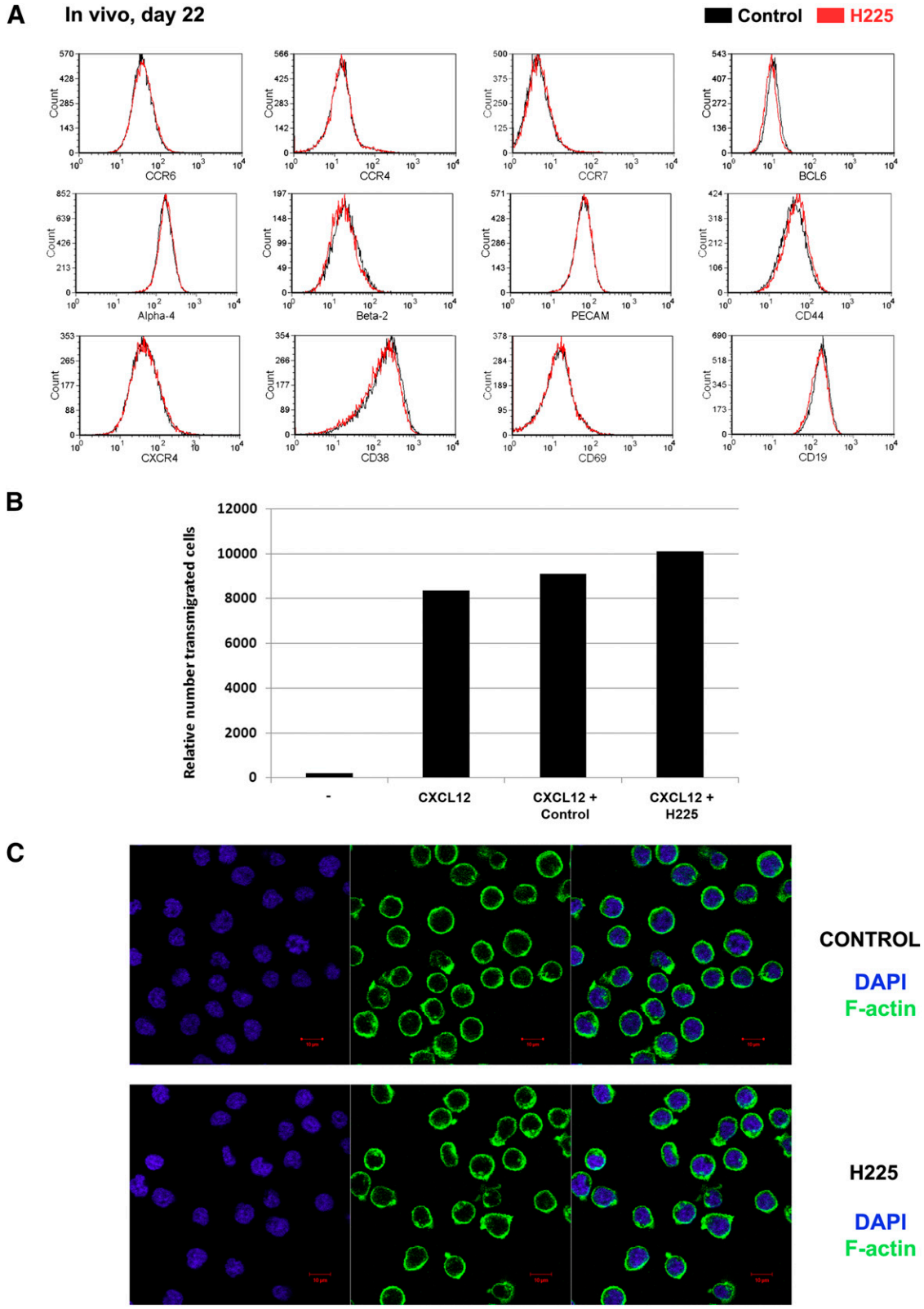

Figure 4. Anti-JAM-C antibody H225 does not affect expression of surface markers, chemotaxis, or actin polymerization. (A) NOD/SCID mice were injected with Jeko-1 cells and treated with control IgG or with H225 antibody, as described in Fig. 2. Jeko-1 cells were detected by flow cytometry with anti-CD19 and anti-CD45 antibodies and analyzed for the expression of cell surface activation markers and adhesion molecules. Black lines: Jeko-1 cells from the mouse treated with control IgG; red lines: Jeko-1 cells from the mouse treated with antibody H225. One representative

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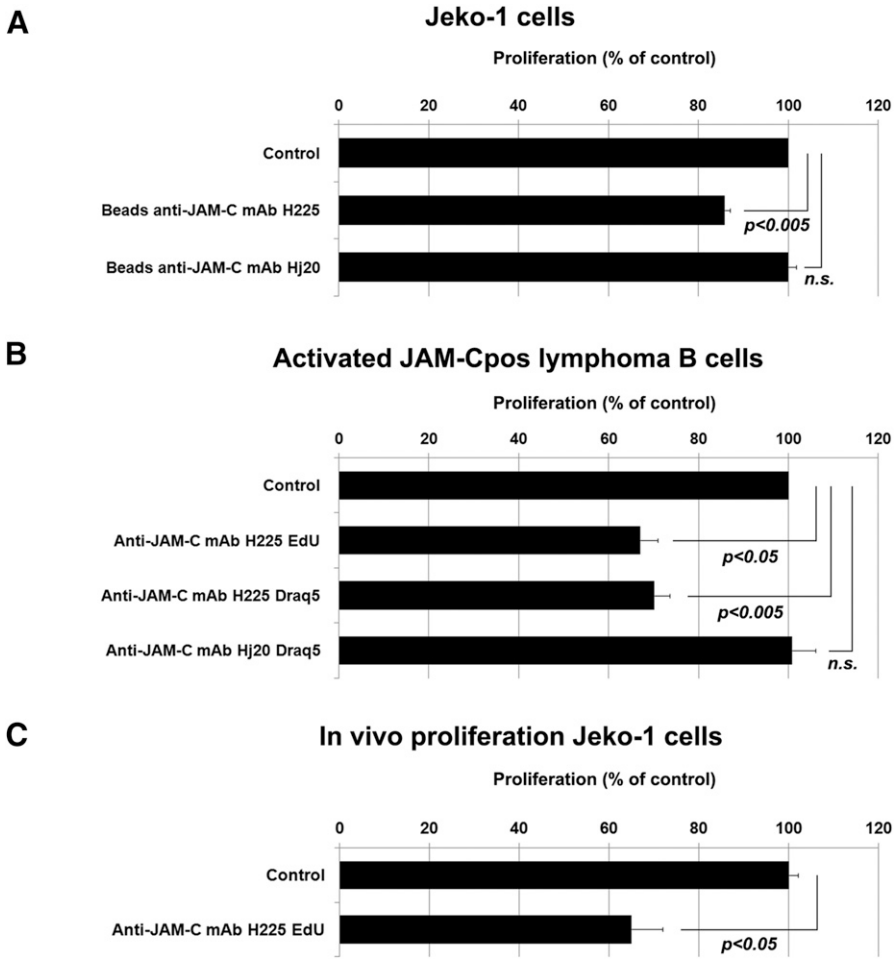

Figure 5. Treatment with anti-JAM-C antibody H225 reduces proliferation of Jeko-1 cells and of primary JAM-C$^+$ lymphoma B cells. (A) Jeko-1 cells were cultured in complete RPMI 1640 medium and incubated with Epoxy-activated beads coated with anti-JAM-C antibody H225, with anti-JAM-C antibody Hj20, or with IgG1 isotype control ($n = 5$). Cell proliferation was evaluated 3 h later by flow cytometry after DRAQ5 staining. (B) Primary lymphoma JAM-C$^+$ B cells were cultured in complete RPMI 1640 containing cytokines (IL-2, -4, and -10), and CD40L and were incubated with the anti-JAM-C antibody H225 or Hj20 or with the IgG1 isotype control ($n = 4$). Cell proliferation was evaluated at day 3 by flow cytometry after DRAQ5 or EdU staining, as indicated. (C) NOD/SCID mice were injected with Jeko-1 cells and treated with control IgG or with H225 antibody ($n = 3$), as described in Fig. 2. At the end of the experiment EdU was injected intravenously, and mice were killed the next day. Organs were collected, and infiltrated Jeko-1 cells from the bone marrow were labeled with anti-CD19 and anti-CD45 antibodies and analyzed for cell proliferation. Results are expressed as means ± SEM of the percentage of proliferation compared to control (100%). Differences between antibody-treated and control cells were determined by ANOVA, followed by Dunnett's post hoc test (A) or Student's *t* test (B, C).

DISCUSSION

In this study, we recorded preclinical data on the efficacy of anti-JAM-C therapy in an MCL mouse model. JAM-C is a surface glycoprotein found at tight junctions of vascular endothelial cells, as well as on human platelets, stem cells, and NK, T, and B cells [6, 4, 14]. JAM-B expressed by vascular endothelial cells has been described as the ligand for JAM-C expressed by hematopoietic cells [6, 15]. Anti-JAM-C blocking antibodies inhibit JAM-B/JAM-C interaction, thereby reducing cell homing [6, 14].

The recirculation and homing of B cells to lymphoid microenvironments is a critical process in the orchestration of the immune response [16, 17]. Normal lymphocyte trafficking and lymphocyte–endothelium interactions are regulated by adhesion molecules, such as the selectins, the integrins VLA-4 and LFA-1 and their ligands VCAM-1 and ICAM-1, and the chemokines [18, 19]. Similar to normal B lymphocytes, malignant lymphoma B cells home to specialized microenvironments favorable to tumor cell survival and growth in lymphoid tissues such as the bone marrow, lymph nodes, or spleen. This migration constitutes a central aspect of the pathophysiology of lymphomas, because it promotes rapid tumor dissemination [20]. Therefore, targeting the molecules, which control the homing of lymphoma cells to their survival niches, could constitute a new treatment strategy for B-cell lymphomas. In a prior publication, we reported the critical importance of JAM-C in controlling the homing of normal and malignant B cells to lymphoid organs by using anti-JAM-C pAbs [6]. Consequently, we generated and analyzed 6 novel anti-JAM-C mAbs that may be more appropriate for therapeutic use. In the present study, we demonstrated that H225, the most efficient anti-JAM-C mAb in the series that we tested, efficiently decreased the homing of malignant B cells to lymphoid organs. Applied in a therapeutic setting, this antibody could therefore help in abolishing lymphoma dissemination to lymphoid niches.

In addition to its effect on homing, H225 treatment eradicated the lymphoma in all organs in NOD-SCID mice when antibody

experiment of 3 is shown. (B) Jeko-1 cells were incubated with IgG control or with anti-JAM-C mAb H225 and added to the top chamber of Transwell culture inserts. Wells contained complete medium, with or without CXCL12. At 1.5 h later, the cells were harvested from the lower well and counted by flow cytometry. (C) Jeko-1 cells were treated with H225 or control IgG for 3 and 24 h mounted on poly-L-lysine-treated slides. Polymerized actin distribution was examined by staining with phalloidin-FITC. One representative experiment of 3 is shown, for the 3 h time point.

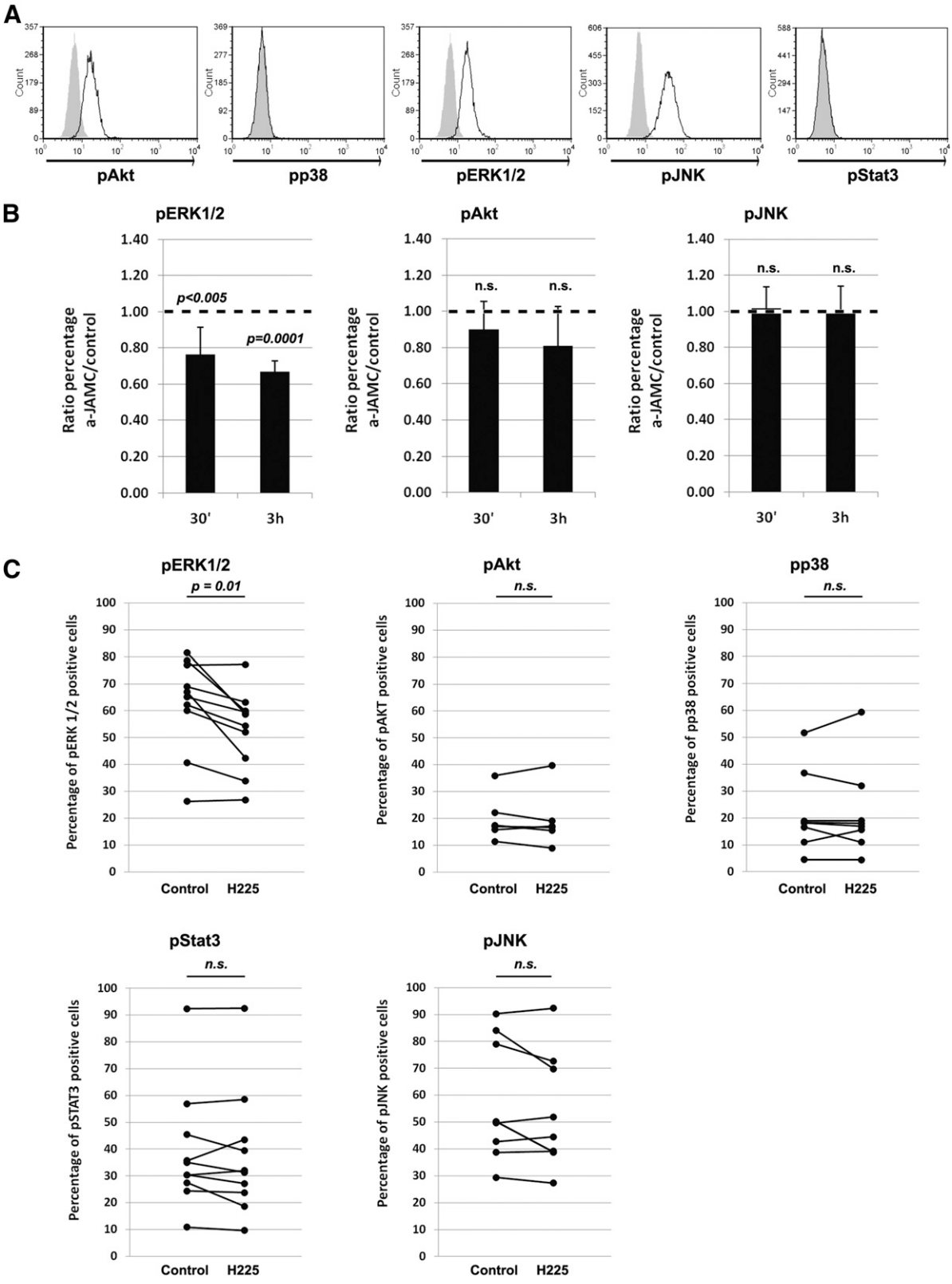

Figure 6. Anti-JAM-C antibody reduces ERK1/2 phosphorylation and does not affect other signaling pathways. (A) Jeko-1 cells were cultured in complete RPMI 1640 medium, and phosphorylation of Akt, p38, ERK1/2, JNK, and Stat3 was evaluated by flow cytometry with phosphospecific mAbs. Unstained cells were used as controls (gray-filled histograms). (B) Jeko-1 cells were cultured in complete RPMI 1640 medium and incubated with anti-JAM-C antibody H225 or isotype control. Phosphorylation of ERK1/2, Akt, and JNK was evaluated 30 min and 3 h later by flow cytometry

(*continued on next page*)

treatment was initiated 6 days after injection of Jeko-1 cells (i.e., after the cells had already homed to the lymphoid organs). Of the different possible mechanisms to explain this finding, we studied the effect of H225 on the expression of other adhesion molecules or chemokine receptors on the lymphoma cells, on B-cell activation status, on actin polymerization, and on B-cell chemotaxis. None of these mechanisms was influenced by H225 treatment, but we observed a direct effect of H225 on the proliferation of Jeko-1 cells. Both in vitro lymphoma cells treated with H225 antibody and cells recovered from lymphoid organs of mice treated for several weeks with H225 showed decreased proliferation rates. This observation is in concordance with the fact that tight-junction proteins not only possess adhesive roles, but are also crucial components of the signaling pathways that regulate proliferation and differentiation [9].

Recent studies similarly have shown that targeting other adhesion molecules such as CD44 or EpCAM, can influence cell proliferation. For instance, anti-CD44 antibodies efficiently inhibited the proliferation of acute myeloid leukemia cells [21, 22]. Although p27 has been implicated, the signaling pathways regulating this inhibition are still largely unknown. EpCAM, which is overexpressed in several epithelial cancers [23], has also been found to be involved in proliferation. In JAM-C$^+$ B-cell lymphomas, particularly MCL, reducing proliferation via binding of JAM-C may therefore be of critical importance in preventing lymphoma growth.

The signaling cascades that initiate and regulate proliferation within cells are composed of phosphoproteins and the kinases and phosphatases that interact with them [24, 25]. We used intracellular phosphospecific flow cytometry [26] to investigate whether anti-JAM-C antibodies modulate these activated signaling molecules in MCL Jeko-1 cells. Indeed, antibody H225 reduced ERK1/2 phosphorylation without affecting other phosphoproteins. To confirm our findings on the Jeko-1 lymphoma cell line, we studied the phosphorylation of primary malignant JAM-C$^+$ B cells. In agreement with the Jeko-1 data, lymphoma B cells treated with anti-JAM-C antibody also showed a decrease in ERK1/2 phosphorylation. Altogether, these data suggest that the therapeutic effects of anti-JAM-C antibodies are not restricted to the prevention of lymphoma cell homing, but could also imply a decrease in ERK1/2 phosphorylation, associated with reduced cell proliferation.

The reduction in tumor growth with H225 is more important than the previously reported effect of anti-JAM-C pAbs [6]. This finding points to the specificity of 1 JAM-C epitope and may allow selective targeting of the molecule for therapy. Anti-tumor efficacy of molecules interfering with the migration of malignant cells, alone or in combination with other therapies, is currently being evaluated for the treatment of lymphomas and other hematologic malignancies. Several studies have already reported antitumor activity of migration inhibitors in lymphoma or leukemia mouse models [27–32]. Some of these molecules have been developed for their use in clinics and are currently under investigation in patients. For instance, A6, a CD44 binding peptide, has demonstrated efficacy and an excellent safety profile in clinical phase 1 and 2 trials in gynecologic cancers, and a phase 2 trial in CLL has started recently (NCT02046928). The combination of plerixafor, a CXCR4 inhibitor, with classic therapies is also being tested in patients with CLL or small lymphocytic lymphoma (NCT00694590) [33] and in patients with acute myeloid leukemia (NCT01027923, NCT00512252, NCT00990054, and NCT01160354). Agents inhibiting B-cell receptor signaling are another current focus of therapeutic development, showing impressive clinical results [34–36]. These molecules also inhibit B-cell migration, homing, and cell adhesion, thereby preventing malignant cells from interacting with survival and growth factors provided by the microenvironment [34].

In conclusion, the data demonstrate that our anti-JAM-C mAbs reduce JAM-C$^+$ lymphoma cell proliferation and homing to supportive lymphoid microenvironments. Treatment with the antibody H225 abolished lymphoma development in the Jeko-1 MCL model in NOD/SCID mice. Therefore, combined treatment of anti-JAM-C antibodies with current therapies could become an attractive approach to improving anti-tumor activity in JAM-C$^+$ lymphoma.

AUTHORSHIP

C.D. and A.V.K. performed experiments and acquisition of data; C.D. and A.V.K performed the statistical analyses; C.D., B.A.I. and T.M. designed the study; C.D., A.V.K., B.A.I., and T.M. wrote and reviewed the manuscript; and B.A.I. and T.M. supervised the study. All authors revised the paper and agreed to its content.

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DISCLOSURE

The authors declare no competing financial interests. A patent application for anti-JAM-C antibody 225.3 has been submitted (PCT/US2013/066534).

with phosphospecific mAbs. Results show means ± SEM of the ratio of phosphorylated cells treated with anti-JAM-C/phosphorylated cells treated with isotype control (no difference, ratio = 1) from 6 experiments. (C) Primary lymphoma JAM-C$^+$ B cells were cultured in complete RPMI 1640 containing cytokines (IL-2, -4, and -10) and CD40L and were incubated with anti-JAM-C antibody H225 or isotype control ($n = 10$). Phosphorylation of ERK1/2, Akt, p38, Stat3, and JNK was evaluated 15 min later by flow cytometry with phosphospecific mAbs. Of note, the phosphorylation of every protein was not always measurable for each patient in the cohort. Differences between antibody treated and control cells were determined by ANOVA followed by Dunnett's post hoc text (A) or Student's t test (B).

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