Clever-1/Stabilin-1 Controls Cancer Growth and Metastasis

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Abstract

Purpose: Immunosuppressive leukocytes and vasculature are important host cell components regulating tumor progression. Clever-1/Stabilin-1, a multifunctional scavenger and adhesion receptor, is constitutively present on a subset of type II macrophages and lymphatic endothelium, but its functional role in cancer is unknown.

Experimental Design: Here, we generated full Clever-1 knockout mice and cell-specific ones lacking Clever-1 either on macrophages or endothelium. We also used anti-Clever-1 antibody therapy to treat B16 melanoma and EL-4 lymphoma.

Results: Clever-1–deficient mice had smaller primary and metastatic tumors than wild-type (WT) controls. Growth of primary tumors, but not of metastases, was attenuated also in mice lacking Clever-1 selectively in macrophages or in vascular endothelium. Anti-Clever-1 antibody treatment inhibited tumor progression in WT mice. Both genetically and therapeutically induced absence of functional Clever-1 led to diminished numbers of immunosuppressive leukocyte types in tumors. Functionally Clever-1–mediated binding of immunosuppressive leukocytes to the intratumoral blood vessels aberrantly expressing Clever-1, and tumor cell traffic via the lymphatics. The antibody therapy did not aggravate autoimmunity.

Conclusion: This work identifies Clever-1 in type II macrophages and in tumor vasculature as a new immunosuppressive molecule in cancer. Our finding that Clever-1 supports binding of tumor-infiltrating lymphocytes to tumor vasculature increases our understanding of leukocyte immigration to tumors. The ability of anti-Clever-1 antibody treatment to attenuate tumor progression in WT mice in vivo is therapeutically relevant. Thus, Clever-1 may be an emerging new target for modulating immune evasion and lymphatic spread in cancer. Clin Cancer Res; 1–13. ©2014 AACR.

Introduction

The ability to invade, induce angiogenesis, and avoid immune destruction are important hallmarks of cancer cells (1). Tumor cells typically invade locally through the extracellular matrix. The cells, which can intravasate into the preexisting or tumor-induced neoangiogenic blood or lymphatic vessels, can form metastases in draining lymph nodes (LN) and at distant sites. Because of differences in the endothelial and vessel wall structure, lymphatic vessels likely offer pathways of lower resistance for migrating cells, which is associated with the fact that approximately 80% of metastasizing tumors preferentially spread via the lymphatic system (2).

In addition to the intrinsic properties of tumor cells and the vasculature, the progression of cancer is also dependent on the quality and quantity of antitumor immune responses (1). Lymphocytes continuously patrol through the blood, lymphoid tissues, and lymphatic vasculature during cancer immune surveillance. Proinflammatory and cytotoxic immune responses are useful in limiting tumor progression, whereas anti-inflammatory immune cell types often paradoxically promote tumor growth. For instance, regulatory T cells (Treg) and type II macrophages can cause immunosuppression, which is one of the main obstacles in successful cancer treatment (3–5). The same cell types also produce multiple proangiogenic molecules and thereby contribute to the angiogenic switch in cancer (6).

Clever-1/Stabilin-1, also known as Feel-1, is a multifunctional molecule conferring scavenging ability on a subset of type II macrophages (5, 7–10). In these cells, it is involved in receptor-mediated endocytosis and recycling, intracellular sorting, and transcytosis of altered and normal self-components (11). Although other leukocyte types (granulocytes and lymphocytes) are Clever-1–negative, it is also constitutively expressed on afferent and
Translational Relevance

Human cancers with high number of Clever-1–positive macrophages are associated with poor prognosis in advanced cancers. However, its mode of action in controlling cancer behavior and potential to use it as a therapeutic target has remained unknown. In this work, we have for the first time demonstrated the immunosuppressive role of Clever-1 in cancer behavior using gene-targeted mice. Most importantly, we have shown that cancer growth and metastases can be efficiently prohibited with an antibody therapy against Clever-1 without any obvious side effects. The inhibitory effects of anti-Clever-1 antibody therapy on progression of primary and metastatic tumors suggest that this molecule is a new immunomodulatory target for cancer immunotherapy. Because it has a unique mode of action, it may have potential benefits in comparison with currently available immunomodulating drugs.

efferent lymphatic endothelial cells, on sinusoidal endothelial cells in the liver and spleen, and on high endothelial venules (8, 12–14). Moreover, upon inflammation, it can be induced on blood vessel endothelium, where it mediates the trafficking of lymphocytes, granulocytes, and monocytes from the blood into the inflamed tissue (13, 15–17).

Clever-1–positive lymphatics and macrophages are found in human cancers and high number of Clever-1–positive macrophages is associated to shorter disease-specific survival in colorectal cancers of advanced stage (15, 18). This work was designed to elucidate the role of Clever-1 in tumor immunity and in tumor cell trafficking, and to test whether it can be used as a therapeutic target in preclinical settings. We generated universal and cell-type selective Clever-1–deficient mice to analyze tumor growth, lymphatic spread, and antitumor immune responses. We also therapeutically targeted Clever-1 by antibodies during tumor growth in wild-type (WT) mice. The results showed that Clever-1 controls tumor progression by mediating leukocyte-subtype selective entrance of tumor-infiltrating leukocytes (TIL) from the blood into the tumor.

Materials and Methods

Animals

Six- to 12-week-old C57BL/6 WT mice were used in antibody experiments. Clever-1 full knockout (Clever-1<sup>−/−</sup>) mice, mice lacking Clever-1 in monocytes/macrophages (Clever-1<sup>−/−</sup>/Lyz2-Cre<sup>(Cre)</sup>/<sup>(Cre)</sup>, called hereafter MACROclever<sup>−/−</sup>), and mice without Clever-1 in vascular endothelium (Clever-1<sup>−/−</sup>/Tie2-Cre<sup>em(Cre)</sup>/<sup>(Cre)</sup>, called hereafter ENDOclever<sup>−/−</sup>) and their WT littermates as controls (as recommended to be a requirement in mouse studies; ref. 19) were used in C57BL/6N;129Sv mixed background. In certain experiments, NADPH oxidase–deficient mice (Ncf<sup>mut</sup>) and New Zealand white (NZW) rabbits were used.

The Clever-1/Stab-1 conditional targeted allele, with recombination sites (loxP) before and after exon 1, was constructed by recombination technology. Conditional Clever-1<sup>−/−</sup> mice were generated by homologous recombination in mice. Clever-1<sup>−/−</sup> mice were bred to CAG-Cre (20), Tie2-Cre (21), and Lyz2-Cre (22) mice, to delete exon 1 of the Clever-1/Stab-1 locus in all cells, vascular endothelial, or myeloid compartments (neutrophils are inherently Clever-1–negative), respectively (Fig. 2A and Supplementary Materials and Methods).

All animal studies were done in adherence with the rules and regulations of The Finnish Act on Animal Experimentation (62/2006), performed in compliance with the 3Rs principle and accepted by the local Committee for Animal Experimentation (Animal licence number 3791/04.10.03/2011).

Tumor cell lines

KCA, a human lymphoblastoid cell line, was a kind gift from E. Engleman (Stanford University, Stanford, CA) in 1984 and its human origin was authenticated in 2011 by using antibodies against human antigens. Mouse B16-F10-luc-G5 melanoma cell line containing a luciferase construct and mouse EL-4 T lymphoma were purchased from Xenogen in 2008 and their authentication is based on color (melanoma is black) and their luciferase activity in each experiment. Tumor cells were cultured in RPMI-1640 (KCA and EL-4) and MEM/HBSS (B16 melanoma; HyClone) supplemented with 10% FBS (Invitrogen, Gibco), nonessential amino acids (Biological Industries), 200 mmol/L I-glutamine (B10 Whittaker), 1 mmol/L sodium pyruvate (Invitrogen, Gibco), and MEM vitamin solution (Invitrogen, Gibco).

Cancer models

B16-F10-luc-G5 melanoma cells (4 × 10<sup>5</sup> cells) or EL-4 lymphoma cells (5 × 10<sup>6</sup> cells) in 30 μL of RPMI (GIBCO) were injected subcutaneously into the left ear or in certain experiments (1 × 10<sup>6</sup> cells in 20 μL) into the footpads of mice. Ear tumors metastasized to the cervical LNs, whereas the footpad ones spread to popliteal LNs. Tumor growth was assessed by luciferase bioluminescence measurements twice a week, using a previously described methodology (23) and also by an electronic caliper (Mitutoyo). The volume of the tumor was calculated according to the formula V = πd<sup>2</sup>/6 × (shortest diameter)<sup>2</sup> × (longest diameter) as described previously (24).

The popliteal LN metastases were visually scored at the end of the experiment as follows: 0, no visible metastasis; 1, metastases (black spots) encompassing less than half of the LN surface; 2, metastases covering more than 50% but less than 100% of LN; 3, whole LN black, but still of normal size; and 4, enlarged, fully black LN.

The antibody treatments in melanoma-bearing WT mice were done with anti-Clever-1 (originally a kind gift from S. Goerdt [Department of Dermatology, Venereology and Allergology, University Medical Center and Medical Faculty...
Mannheim, Heidelberg University, Mannheim, Germany] and then purchased from Invivo Biotech; ref. 25) or NS-1 control antibody (produced by Invivo Biotech) using prophylactic and therapeutic protocols. In the prophylactic settings, the mice were treated with by subcutaneous injections of the mAbs (50 μg) into the ear 1 day before tumor injection followed by intraperitoneal dosing (100 μg/infec-
tion) starting 1 day after tumor injection and then repeated every third day. In the therapeutic experiments, no local antibodies were given and the intraperitoneal antibody therapy was first started 3 days after B16 melanoma cell injections (1 × 10^6 cells in 20 μL).

**Ex vivo adhesion assays**

TILs were isolated from melanomas grown in nontreated WT mice using previously described methodology (26). Briefly, the isolated tumors were minced to small pieces and digested with collagenase D (1 mg/mL, +37°C, 40 minutes; in the presence of DNase I). The released cells were purified using anti-CD45-PE antibody (BD Pharmingen) and magnetic cell sorting (Mouse PE selection kit, EasySep; STEMCELL Technologies). CD4^+ cells were separated from the blood of WT mice using the EasySep mouse CD4^+ T-cell preenrichment kit according to the instructions of the manufacturer. Whole blood after erythrocyte lysis was used as a source of monocytes.

Melanomas were collected from anti-Clever-1 and control antibody-treated mice on day 14, snap-frozen, and sectioned. The ex vivo adhesion assays were performed as previously described (27). Briefly, isolated TIL and CD4^+ cells from blood were allowed to bind to vessels in the melanoma sections for 30 minutes at +3°C under rotatory conditions. In the second set of assays, blood leukocytes from tumor-bearing mice were preincubated with anti-Clever-1 or control antibody for 30 minutes and washed twice before application onto the tissue sections. The nonbound cells were then gently decanted off from the sections, and the adherent cells were fixed in 1% glutaraldehyde. The number of leukocytes bound to tumor vessels was counted under dark-field microscopy. These conditions allowed us to discriminate the TIL (small and phase-bright), tumor-infiltrating myeloid cells (mainly macrophages, which are large and have a ruffled appearance), and blood monocytes (large with a ruffled appearance; ref. 28). At least 100 vessels from each independent tumor in both treatment groups were counted and the average number of leukocytes bound per vessel in control treated mice was used to define 100% adherence.

**Immunohistochemistry**

Acetone-fixed frozen sections of the primary tumors and metastases of the mice inoculated with melanoma cells were stained with rat mAbs against macrophage mannose receptor (MRC), MRSD3, a marker for type II macrophages, (a kind gift from L. Martinez-Pomares, University of Nottingham, Nottingham, United Kingdom; ref. 29), PV-1 antigen (blood vessel antigen, Meca-32, a kind gift from E. Butcher, Stanford University), CD31 (a marker of both blood and lymphatic vessels; BD Pharmingen), CD3 (BD Pharmingen), CD8 (Caltag), SPARC (R&D Systems), and rabbit polyclonal antibody against LYVE-1 (a lymphatic endothelium-specific marker; Reliatech), or with a negative control mAb (Hermes-1 against human CD44) or normal rabbit serum. FITC-conjugated anti-rat Ig (Sigma) or FITC-conjugated anti-rabbit Ig (Sigma) diluted in PBS containing 5% normal mouse serum was used as the second-stage antibody. Tumor tissues, skin, metastases, and LN sections were also stained using biotinylated anti-Clever-1 (1.26) followed by Streptavidin–Alexa Fluor 546. Alternatively, 9–11 (rat anti-human Clever-1, which cross-reacts with the mouse homolog; ref. 30) or 3–372 (mouse anti-human Clever-1, which cross-reacts with the rabbit homolog) followed by FITC-conjugated anti-rat Ig or mouse Ig was used (13). For double stainings, anti-LYVE-1 or anti-PV-1 followed by Alexa Fluor 546–conjugated anti-rabbit IgG (Invitrogen) or Alexa Fluor 546–conjugated anti-rabbit IgG (Invitrogen), respectively, was used together with Alexa Fluor 488–

**Coculture experiments**

Blood monocytes and peritoneal macrophages from WT mice were added to the upper compartments of Transwell (transparent polyester membranes, pore size 0.4 μm; Corning) in medium containing 20 μg/mL anti-Clever-1 (1.26) antibody or negative control antibody, and B16 melanoma (50,000 cells) were simultaneously plated to the lower compartments. After 3 and 7 days of coculturing, the methanol-fixed membranes with adherent monocytes/macrophages were stained with SD3 against MR, 9–11 against Clever-1, and negative control antibody followed by FITC anti-rat Ig second stage. Percentages of positive cells were counted under a fluorescence microscope (Olympus BX60).

**Immune array qPCR**

Isolated CD45^+ TIL and nonhematopoietic CD45^+ cells from B16 melanomas from anti-Clever-1 or control antibody treated mice (n = 6) were pooled in groups of two. Total RNA was isolated (Nucleo-Spin RNAII Total RNA Isolation Kit (Macherey-Nagel)) and reverse-transcribed using iScript cDNA Synthesis Kit (BioRad). Equal amounts of samples were loaded into TaqMan Mouse Immune Array Microfluidic Cards (Applied Biosystems).
and run using a 7900HT Fast Real-Time PCR System (Applied Biosystems) in the Finnish Microarray and Sequencing Center, Center for Biotechnology (Turku, Finland). The results were normalized using 18S RNA as an endogenous control. The results were analyzed with SDS 2.3 and DataAssist v3.0 software using relative quantification (RQ).

**Tumor cell migration via lymphatics**

Rabbits were treated with 2 mg/kg of 3–372 or control antibody intravenously on days −1 and 0. CFSE-labeled KCA lymphoma cells (40 × 10^6 cells labeled with 0.5 µmol/L CSFE for 20 minutes at 37°C followed by three washings) together with additional 0.5-mg dose of antibodies were injected subcutaneously into the footpads at day 0. After 24 hours, the popliteal LNs were collected and cell suspensions were analyzed by flow cytometry (FACSCalibur; BD Biosciences) to quantify the numbers of immigrated CFSE-positive cells.

**Statistical analyses**

The two-tailed Student test and Mann–Whitney U test were used. P values < 0.05 were considered significant.

**Results**

**Induction of Clever-1 in tumor vasculature and macrophages**

In normal WT mice, Clever-1 is expressed in lymphatics and faintly in high endothelial venules but is practically absent on flat-walled vessels and macrophages in LNs (Fig. 1A). The expression pattern of Clever-1 was altered in B16 melanoma-bearing mice. The majority of the intratumoral vessels in primary tumors and LN metastases were enlarged with a widely open lumen and, unlike the normal flat-walled vessels, they expressed Clever-1 (Fig. 1B). Moreover, a subset of Clever-1–positive macrophages not seen in normal LNs was detected in the primary tumors and LN metastases (Fig. 1C). All Clever-1–positive macrophages were MRC–positive.
(a prototype marker for type II macrophages), but only about 50% of MRC-positive macrophages coexpressed Clever-1. Tumor cells induced Clever-1 expression in macrophages, because Clever-1-positive macrophages were not found in enlarged draining LNs after a 17-day subcutaneous OVA immunization (Supplementary Fig. S1). In kinetic analyses of tumors on days 3, 6, and 10, the first MRC-positive macrophages were seen in day 6 samples, whereas Clever-1 was first detected on macrophages in day 10 samples. Clever-1-positive monocytes were also present in the blood of tumor-bearing mice. These results indicate that at least two different subpopulations of MRC-positive macrophages (MRC+/Clever-1+ and MRC+/Clever-1−) exist in cancer but the former is not induced during non-cancer-related immune response.

To study, whether the lack of Clever-1 has an impact on tumor development and metastasis, we generated Clever-1 full knockout mice (Clever-1−/−), mice lacking Clever-1 in macrophages (MACROclever−/− mice), and mice lacking this molecule in blood vessel endothelium (ENDOclever−/− mice; Figs. 2A and Supplementary Fig. S2). All cell types in tumors of Clever-1−/− mice completely lacked Clever-1 expression, as expected (Fig. 2B). In tumors of MACROclever−/− mice, Clever-1 expression in the lymphatics and blood vessels was comparable with tumor-bearing WT mice, whereas almost all Clever-1-positive macrophages had disappeared (Fig. 2C). Tumors of ENDOclever−/− mice, on the other hand, did not have visible Clever-1 expression on PV-1-positive blood vessels but macrophages and the
majority of lymphatic vessels brightly expressed Clever-1 (Fig. 2D).

Retarded tumor growth and metastatic dissemination in absence of Clever-1

The primary B16 tumors remained significantly smaller in Clever-1−/− mice than in WT mice and the same trend was seen in MACROclever−/− mice when compared with WT mice on day 14 (P = 0.057; Fig. 3A). However, the sizes between MACROclever−/− tumors were significantly smaller on day 9/10 than the WT tumors (P = 0.009). In the next experiment, primary tumors of also ENDOclever−/− mice tended to remain smaller than those of WT mice (Fig. 3B) and were comparable with the size of tumors in Clever−/− mice (see Fig. 3A for comparison). If the size of tumors in ENDOclever−/− mice was compared with size of all WT tumors in this set of experiments, the difference was statistically significant (P = 0.046) at the end of the experiments (day 14). In contrast, metastases remained small only in the full Clever−/− mice while the size of the metastases in the cell type–specific knockouts did not differ from those of the WT mice (Fig. 3C). Thus, Clever-1 is needed for normal progression of primary melanoma tumors in vivo both on macrophages and vascular endothelium. Moreover, because lymphatics in ENDOclever−/− and MACROclever−/− mice are Clever-1–positive and full Clever-1−/− lacking Clever-1 also on lymphatics have only small metastases, Clever-1 in lymphatics seems to be important in mediating the spread of tumor cells into the draining LNs.

As the future aim is to test the potential of Clever-1 as a target in human malignancies, we used another cancer model to test whether anti-Clever-1 mAb interferes with cancer cell migration via the lymphatics. For these experiments, we selected rabbit as a model, as we then could use anti-human Clever-1 mAb 372, which cross-reacts with rabbit Clever-1 (Supplementary Fig. S3A), and Clever-1–negative human lymphoma cells (Supplementary Fig. S3B). The blockade of lymphatic Clever-1 with this cross-reacting mAb in this model efficiently blocked the trafficking of lymphoma cells from the footpad to the draining popliteal LNs (Fig. 3D). This supports the notion that lymphatic Clever-1 may promote metastasis formation.

Reduced tumor growth during antibody therapy

To test whether Clever-1 can be used as a therapeutic target in cancer, we next used function-blocking antibody against mouse Clever-1. We injected B16 melanoma and EL-4 lymphoma cells, both of which are Clever-1–negative (Supplementary Fig. S4A and S4B), subcutaneously into pinna of ears to allow in vivo imaging of metastasis in the neck. In the B16 model, both the primary tumors and the metastases were about 70% smaller in mice treated with anti-Clever-1 antibody in comparison with the control-treated animals, when the treatment was started before the tumor cell injections (Fig. 4A and B and Supplementary Fig. S4C).

In the EL-4 lymphoma model, tumor growth was observed in 9/11 control-treated and 9/10 anti-Clever-1–treated mice. Among the mice with detectable tumor growth, the primary tumors remained significantly smaller in the anti-Clever-1–treated group (Fig. 4C). The EL-4 metastases in draining LNs were on average 49% smaller on day 11 (P < 0.01) and 26% smaller on day 14 in the anti-Clever-1–treated group than in the control antibody-treated group, but this difference did not reach statistical significance (Fig. 4C).

To evaluate the efficacy of Clever-1 blockade in a clinically relevant therapeutic setting, we then let the B16 tumors grow for 3 days before starting the antibody therapy. In this model, the anti-Clever-1 antibody treatment also led to a statistically significant reduction in primary tumors and metastases on day 20 (Fig. 4D). Thus, prophylactic and therapeutic neutralization of Clever-1 by mAbs attenuates tumor growth in vivo.

Clever-1 is needed for intratumoral accumulation of type II macrophages and FoxP3–positive T cells but not for neoangiogenesis

Next, we analyzed whether decreased tumor growth in the absence of Clever-1 would be associated with altered tumor neo(lymph)angiogenesis or leukocyte infiltration. In the tumors of Clever-1−/− mice, the density of F4/80 and MRC-positive macrophages and FoxP3–positive T cells was diminished and no difference was found in the numbers of intratumoral CD3+ and CD8+ T cells or in CD31+ vessels (Fig. 5A). Number of MRC-positive macrophages and FoxP3–positive cells were also reduced both in the primary tumors and metastases of anti-Clever-1 mAb-treated WT mice in comparison with control mAb-treated mice (Fig. 5B). Moreover, by comparing tumors of overlapping sizes in anti-Clever-1 and control-treated groups, we found that the diminished numbers of intratumoral type II macrophages subsequent to the antibody therapy was not dependent on the size of the tumors (Supplementary Fig. S5A). The reduction of MRC- and FoxP3–positive cells was selective because the numbers of intratumoral CD3+ and CD8+ cells were comparable in both treatment groups (Fig. 5B).

Although Clever-1 has been reported to play a role in angiogenesis in vitro (7) the numbers of intratumoral blood vessels (CD31+ and PV-1–positive) and lymphatics (CD31+ and PV-1–negative) were comparable after anti-Clever-1- and control antibody treatments in WT mice (Fig. 5B). Together, these data thus show that the number of regulatory immune cell types is diminished subsequent to targeting Clever-1, but both the blood and lymphatic vasculature remain unchanged (Fig. 5).

Anti-Clever-1 mAb treatment does not alter scavenging of SPARC or macrophage polarization

As Clever-1 on macrophages mediates the uptake of SPARC (9), an extracellular matrix molecule regulating tumor growth, the amount of SPARC could contribute to the reduced tumor growth in anti-Clever-1–treated mice. However, the expression of SPARC was found to be similar in tumors of anti-Clever-1– and control antibody-treated mice (Supplementary Table S1).
Because anti-Clever-1 treatment significantly decreased the number of MRC-positive macrophages (Fig. 5B) the antibody might lead to the depletion of MRC–Clever-1+ macrophages by antibody-dependent cell-mediated cytotoxicity or complement activation. However, this was not the case because after anti-Clever-1 and control mAb administration, similar proportion of intratumoral type II macrophages in primary tumors coexpressed Clever-1 (51.7% ± 5.9%, n = 5 and 52.2% ± 6.4%, n = 4 of type II MRC-positive macrophages were Clever-1–positive in anti-Clever-1 mAb and in control mAb-treated mice, respectively).

To analyze the potential effect of anti-Clever-1 mAb on macrophage polarization, we used in vitro polarization of blood monocytes and peritoneal macrophages from WT mice. Presence of B16 melanoma cells polarized the monocytes/macrophages toward type II cells significantly better than the culture medium alone (Supplementary Fig. S5B).
Figure 4. Anti-Clever-1 antibody treatment diminishes primary tumor growth and metastases. A, B16-luc melanoma cells were injected subcutaneously into the ear. Growth of the primary tumor and the development of metastases were assessed by bioluminescence. Relative size (mean ± SEM) of the primary tumor after 10 and 14 days in the two treatment groups (n = 12 in both groups, combined results of two experiments) and relative size (mean ± SEM) of metastases at the end of the experiment (day 14). The size (assessed by luciferase bioluminescence counts) of the primary tumor and metastases in the control-treated group is 1.0 by definition. Results of one experiment with raw values are shown in the inset. B, examples of tumors in animals treated with anti-Clever-1 or control antibody. White arrows point to the injection site (primary tumor) and yellow arrows to the neck metastases. Note that one anti-Clever-1 antibody–treated mouse does not have a detectable tumor at the site of injection and the other does not have neck metastases. C, kinetics of EL-4 T-cell lymphoma growth in anti-Clever-1- and control antibody–treated animals. Only animals with tumor growth were included (9/11 in control antibody–treated and 9/10 in anti-Clever-1–treated groups). The combined data of two experiments are presented as the relative volume of the tumor and relative bioluminescence counts of the metastases (mean ± SEM). The control-treated group is 1.0 by definition. Results of one experiment with raw values are shown in the inset. D, development of primary tumor and metastases when the antibody treatment was started 3 days after the injection of B16 melanoma cells (day 14, n = 12 in both groups, and day 20, n = 6 in both groups). The data are presented as in A.

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and S5C). Notably, the presence of saturating anti-Clever-1 mAb concentrations had no effect on this process. Together, these data strongly suggest that the antibody treatment does not deplete macrophages and has no direct effect on their M1/M2 polarization.

**Clever-1 mediates binding of TIL to tumor vasculature**

Because Clever-1 is involved in the extravasation of leukocytes to lymphoid organs and into sites of inflammation (17), we tested whether entrance of TIL (or that of their precursors) was inhibited during the antibody therapy. We collected tumors from both anti-Clever-1- and control antibody-treated animals and tested the binding of TIL and peripheral blood CD4+ T cells isolated from nontreated WT mice to vessels in those tumors ex vivo. Both tumor-infiltrating myeloid cells and lymphocytes bound less efficiently to tumor vessels of anti-Clever-1–treated animals. Also, the adhesion of CD4+ blood lymphocytes to the tumor vasculature of Clever-1–treated mice was reduced in comparison with those of control antibody-treated mice (Fig. 6A). Moreover, to test whether monocyte Clever-1 contributes to adherence to the endothelium, we treated blood monocytes and lymphocytes (lymphocytes served as controls as they are Clever-1–negative) collected from tumor-bearing mice with anti-Clever-1 mAb in vitro before the adhesion assay. Anti-Clever-1 mAb inhibited monocyte binding to tumor vessels by 70%, whereas no inhibition was seen in lymphocyte binding (Fig. 6B). Thus, anti-Clever-1 therapy may prevent leukocyte entrance into the tumors by targeting Clever-1 both on the vascular endothelium and monocytes and is well in line with the results obtained with MACROclever+/− and ENDOfClever+/− mice.

**Anti-Clever-1 treatment is responsible for increased immune activation and apoptosis in tumors**

To analyze whether decreased numbers of immunosuppressing leukocyte types in tumors after anti-Clever-1 treatment associates with increased antitumor immune response, we performed qPCR immune arrays. Within the TIL (CD45+) population (Fig. 6C), anti-Clever-1 treatment increased the expression of many activation markers (e.g., proinflammatory CCL3, IL1 and IL6). In the non-hematopoietic cell population of the tumors (CD45− cells, mainly containing tumor cells together with some non-hematopoietic normal cells such as endothelial cells), inflammation-induced endothelial markers such as E- and P-selectin were decreased (Fig. 6D). In the CD45− population, the expression of proapoptotic Smad7 was increased and the expression of the antiapoptotic marker Bcl2-like protein 1 (Bcl2l1) was decreased. The enhanced apoptosis of the tumors subsequent to anti-Clever-1 therapy was also confirmed by in situ apoptosis detection. The mean number of apoptotic cells in tumors of anti-Clever-1 antibody–treated mice (n = 4) was 46.4 ± 4.8/high-power field (HPF) and 15.7 ± 5.7/HPF in tumors of control antibody-treated mice (n = 5; P = 0.005).

**Anti-Clever-1 therapy does not aggravate autoimmune inflammations**

As anti-Clever-1 treatment leads to immune activation in cancer, it might aggravate inflammation in other settings. However, in neutrophil- and lymphocyte-dominated models of arthritis, induced by anti-collagen II antibodies (CAIA) and collagen (CIA), respectively, the anti-Clever-1 mAb therapy did not modify the disease course (severity or incidence). Similar results were also obtained in arthritis experiments performed with Ncf1-mutated mice, which normally display a severe form of CIA (Supplementary Fig. S6). Thus, anti-Clever-1 antibody treatment appears to have different immunomodulatory effects in inflammation related to cancer and autoimmune diseases.

**Discussion**

In this work, we generated conditional Clever-1–deficient mice to study the role of this multifunctional molecule in tumor progression in vivo. We found that the progression of melanoma tumors was attenuated in the absence of Clever-1 from all cells, from macrophages, or from vascular endothelial cells. Prophylactic, and most importantly, therapeutic treatment of WT mice with anti-Clever-1 mAbs inhibited tumor progression. Clever-1 was induced in tumor vasculature and in monocytes of tumor-bearing mice. During tumorigenesis, Clever-1 likely contributes to the extravasation of TILs, because blocking of endothelial and monocyte Clever-1 impaired adhesion of blood-borne leukocytes to vascular endothelium.

Tumors in Clever-1–deficient mice and in WT mice treated with anti-Clever-1 mAbs had lower numbers of MRC-positive macrophages than tumor-bearing control mice. MRC is regarded as one of the best phenotypic markers of type II macrophages, which are known to play profound immunosuppressive functions in tumors (31). These cells most likely enter the tumors as monocytes from blood, and then polarize to type II macrophages under the local influence of tumor microenvironment, although differentiation...
Inducible Clever-1 on tumor vasculature and blood monocytes of tumor-bearing mice mediates leukocyte-endothelial cell interaction, and changes in TIL populations are reflected in their inflammatory status. A, binding of two major populations of TIL, myeloid cells and lymphocytes, and CD4+ cells from the blood (both from untreated melanoma-bearing WT mice) to vessels in melanomas obtained from mice treated in vivo with anti-Clever-1 (n = 3) or control antibody (n = 3) analyzed using ex vivo–frozen section assays. The number of leukocytes bound to control antibody-treated melanomas was set as 100% by definition and the data are presented as the mean ± SEM of control binding. B, binding of blood monocytes and lymphocytes from tumor-bearing mice to tumor vasculature using ex vivo–frozen section assays. Blood leukocytes were preincubated with anti-Clever-1 or control antibody and washed before the assay. Analyses were done as in A. Note that in A the Clever-1 blocking is on endothelium and in B on leukocytes (monocytes). Binding of monocytes (large ruffled, two pointed out by arrows) and lymphocytes (small, two indicated by open arrows) to tumor vasculature (surrounded by a dashed line) in an ex vivo–frozen section assay is shown as an example on the right. C and D, mRNA expression of the indicated target genes in CD45+ cells of anti-Clever-1 antibody-treated mice. The corresponding expression levels in the control antibody-treated mice are set to 1.0 (dotted line). Only the genes with RQ values well (33). The anti-Clever-1 mAb did not deplete target antigen-positive macrophages in vitro. Our in vivo results clearly showed that the intratumoral monocytes/macrophages need more than 3 days to differentiate to MRC-positive macrophages in melanoma is compatible with the idea of differentiation-induced induction of Clever-1 on type II macrophages. Our in vivo results clearly showed that the intratumoral monocytes/macrophages need more than 3 days to differentiate to MRC-positive macrophages in vivo. Importantly, anti-Clever-1 antibody did not interfere with the polarization process. Also, the kinetics of appearance of MRC- and Clever-1–positive macrophages in melanoma is compatible with the idea of differentiation-induced induction of Clever-1 on type II macrophages. Our in vivo results clearly showed that the intratumoral monocytes/macrophages need more than 3 days to differentiate to MRC-positive macrophages. The anti-Clever-1 mAb treatment with anti-Clever-1 does not cause vascular damage or affect leukocyte counts (17). This is of course notwithstanding the possibility that Clever-1 ligation might alter the functional responsiveness (e.g., immunosuppressive functions) of targeted macrophages.

Type II macrophages also induce Tregs both via direct contacts (34) and via soluble mediators (32). For example, typical high IL10 production of type II macrophages leads to the expansion of Tregs, which, in turn, produce additional suppressive factors (4) and dampen the immune attack against the tumors, including B16 melanomas (35). Thus, anti-Clever-1 mAb therapy may initially lead to diminished numbers of intratumoral type II macrophages. They, in turn, deliver suboptimal signals for maintaining normal numbers of Tregs. This was seen as reduced numbers of FoxP3-positive cells. Slower tumor growth and increased apoptosis after Clever-1 mAb treatment was also supported by qPCR analyses showing an increase in expression of Smad7 and a decrease of Bcl2l1 in the tumor cell–containing population. We postulate that the primary effect of the anti-Clever-1 treatment is on blocking of Clever-1 function, that is,
leukocyte adhesion to Clever-1 molecule on tumor endothelium in agreement with the reported adhesive function of Clever-1 in leukocyte transmigration (15, 16). We found aberrant Clever-1 expression in intratumoral vessels in the melanoma model. The fact that anti-Clever-1 antibody treatment inhibited TIL and blood leukocyte binding to tumor endothelium in in vitro assays supports this interpretation. It was technically not possible to formally verify this hypothesis in vivo, because leukocyte accumulation in the tumor is slow when compared with LNs in an appropriate time window to allow reliable discrimination of homing from local proliferation. Also in humans, Clever-1 is induced both in intratumoral and peritumoral blood vessels and it supports cancer cell binding to tumor vasculature in in vitro assays (15). The numbers of Tregs and type II macrophages, but not other leukocyte subtypes, were reduced in the tumors after Clever-1 mAb treatment. This implies that counter-receptors for Clever-1, which remain to be identified, may be selectively expressed on different leukocyte subtypes.

The results with cell type–specific Clever-1–deficient mice indicate that Clever-1 in tumor endothelium and monocytes/macrophages is decisive in the growth of the primary tumors, whereas Clever-1 in lymphatics contributes to migration of the cancer cells into the draining LNs. The role of Clever-1 on lymphatics is in line with our earlier studies, which have demonstrated the importance of Clever-1 in the trafficking of normal lymphocytes via afferent lymphatics into the draining LNs (17). Here, we used an independent model for analyzing migration of subcutaneously injected cancer cells via the afferent lymphatics to draining LNs and found nearly 70% decrease in the presence of function-blocking anti-Clever-1 mAb. This further strengthens the notion that metastasizing tumor cells may use Clever-1 in lymphatics for trafficking.

In conclusion, we report here aberrant expression of Clever-1 in tumor blood vessels and its central role in binding of TILs. The inhibitory effects of anti-Clever-1 antibody therapy on progression of primary and metastatic tumors suggest that this molecule may be a new immunomodulatory target for cancer immunotherapy. Because anti-Clever-1 treatment, unlike, for example, CTLA-4 inhibitors (36), did not aggravate autoimmune reactions, and because it has a unique mode of action, it may have potential benefits in comparison with currently available immunomodulating drugs.

Disclosure of Potential Conflicts of Interest
M. Salmi and S. Jalkanen have ownership interest (including patents) in Faron Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

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References
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