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

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Running title: Clever-1 regulates cancer progression

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S. Jalkanen and M. Salmi own stocks of Faron Pharmaceuticals.
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 immune-modulatory target for cancer immunotherapy. Since it has a unique mode of action, it may have potential benefits in comparison to currently available immune-modulating drugs.
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 2 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 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 wild-type 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 intra-tumoral 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 2 macrophages and in tumor vasculature as a new immunosuppressive molecule in cancer. Our finding that Clever-1 supports binding of tumor infiltrating leukocytes to tumor vasculature increases our understanding of leukocyte immigration to tumors. The ability of anti-Clever-1 antibody treatment to attenuate tumor progression in wild-type mice *in vivo*, is therapeutically relevant. Thus, Clever-1 may be an emerging new target for modulating immune evasion and lymphatic spread in cancer.
Keywords endothelium; lymphatics; macrophages; migration; vasculature
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 pre-existing or tumor induced neoangiogenic blood or lymphatic vessels can form metastases in draining lymph nodes and at distant sites. Due to 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 anti-tumor immune responses (1). Lymphocytes continuously patrol through the blood, lymphoid tissues and lymphatic vasculature during cancer immune surveillance. Pro-inflammatory 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 and type 2 macrophages can cause immunosuppression, which is one of the main obstacles in successful cancer treatment (3-5). The same cell types also produce multiple pro-angiogenic 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 2 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). While other leukocyte types (monocytes, granulocytes and lymphocytes) are Clever-1 negative, it is also constitutively expressed on afferent and 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 pre-clinical settings. We generated universal and cell-type selective Clever-1 deficient mice to analyze tumor growth, lymphatic spread and anti-tumor immune responses. We also therapeutically targeted Clever-1 by antibodies during tumor growth in wild-type mice. The results showed that Clever-1 controls tumor progression by mediating leukocyte-subtype selective entrance of tumor-infiltrating leukocytes from the blood into the tumor.

Materials and Methods

Animals

Six to twelve weeks old C57BL/6 WT mice were used in antibody experiments. Clever-1 full knockout (Clever-1$^{-/-}$) mice, mice lacking Clever-1 in monocytes/macrophages (Clever-1$^{fl/fl}$Lyz2Cre$^{(Cre)/(Cre)}$; called hereafter MACROclever$^{-/-}$) and mice without Clever-1 in vascular endothelium (Clever-1$^{fl/fl}$Tie2Cre$^{tm(Cre)^+}$; called hereafter ENDOclever$^{-/-}$) and their WT littermates as controls (as recommended to be a requirement in mouse studies (19)) were used in C57BL/6N;129SvJ mixed background. In certain experiments NADPH oxidase deficient mice (Ncf$^{mut}$) 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>fl/fl</sup> mice were generated by homologous recombination in mice. Clever-1<sup>fl/fl</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, CA) in 1984 and its human origin was authenticated in 2013 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 (Alameda, CA) 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, Logan, Utah) supplemented with 10% FBS (Invitrogen, Gibco), non-essential amino acids (Biologial Industries, Haemek, Israel), 200 mM L-glutamine (B10 Whittaker, Walkersville, MD), 1 mM sodium pyruvate (Invitrogen, Gibco), and MEM vitamin solution (Invitrogen, Gibco).
Cancer models

B16-F10-luc-G5 melanoma cells (4 x 10^5 cells) or EL-4 lymphoma cells (5 x 10^6 cells) in 30µl of RPMI (GIBCO) were injected subcutaneously into the left ear or in certain experiments (1x10^6 cells in 20 µl) into the footpads of mice. Ear tumors metastasized to the cervical lymph nodes, whereas the footpad ones spread to popliteal lymph nodes. 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 = \pi/6 \times (shortest diameter)^2 \times (longest diameter) as described (24).

The popliteal lymph node 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 wild-type mice were done with anti-Clever-1 (25) (originally kind gift of S. Goerdt and then purchased from InVivo Biotech, Hennigsdorf, Germany) 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 1d before tumor injection followed by intra-peritoneal dosing (100 µg/injection) starting 1d after tumor injection and then repeated every third day. In the therapeutic experiments, no local antibodies were given and the intra-peritoneal antibody therapy was first started three days after B16 melanoma cell injections (1 x 10^6 cells in 20 µl).
Ex vivo adhesion assays

TIL were isolated from melanomas grown in non-treated 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 min; 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, Stem Cell Technologies). CD4 positive cells were separated from the blood of wild-type mice using the EasySep mouse CD4+ T cell pre-enrichment 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 positive cells from blood were allowed to bind to vessels in the melanoma sections for 30 min at +7°C under rotatory conditions. In the second set of assays, blood leukocytes from tumor bearing mice were pre-incubated with anti-Clever-1 or control antibody for 30 min and washed twice before application onto the tissue sections. The non-bound 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 tumor-infiltrating lymphocytes (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, (28). At least 100 vessels from three independent tumors 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, MR5D3, a marker for type 2 macrophages ((29), a kind gift from L. Martinez-Pomares, University of Nottingham), PV-1 antigen (blood vessel antigen, Meca-32, a kind gift from E. Butcher, Stanford University, CA), 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 lymph node 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, (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 Alexa546-conjugated anti-rabbit IgG (Invitrogen) or Alexa546-conjugated anti-rat IgG (Invitrogen), respectively, was used together with Alexa488-conjugated anti-Clever-1 (9-11). Anti-FoxP3 (a surrogate marker for regulatory T cells from eBioscience) stainings were done from frozen sections fixed with 2% paraformaldehyde, using peroxidase-conjugated rabbit anti-rat Ig (Dako) and 3,3’-diaminobenzidine hydrochloride for visualization.
TACS 2TdT-Blue Label In Situ Apoptosis Detection Kit (Trevigen) was used according to manufacturer’s instructions to detect apoptosis on tumor sections. The numbers of apoptotic cells/HPF (the whole section, 9-24 fields/slide depending on the size of the tumor) were counted.

The stainings were analyzed using Olympus BX60 microscope and cell^D version 2.6 software (Soft Imaging Solutions GmbH). Intensity of SPARC staining was analyzed using Image J software.

Co-culture experiments

Blood monocytes and peritoneal macrophages from WT mice were added to the upper compartments of trans-wells (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 a 3.5 d co-culture, the methanol-fixed membranes with adherent monocytes/macrophages were stained with 5D3 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 non-hematopoetic 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 day -1 and day 0. CFSE-labeled KCA lymphoma cells (40x10^6 cells labeled with 0.5 µM CSFE for 20 min at 37°C followed by 3 washings) together with additional 0.5 mg dose of antibodies were injected subcutaneously into the footpads at day 0. After 24 h, the popliteal lymph nodes were collected and cell suspensions were analyzed by flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA) to quantify the numbers of immigrated CFSE-positive cells.

**Statistical analyses**

Two tailed Student’s 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 lymph nodes (Fig. 1A). The expression pattern of Clever-1 was altered in B16 melanoma bearing mice. The majority of the intra-tumoral vessels in primary tumors and lymph node 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 lymph nodes was detected in the primary tumors and lymph node metastases (Fig. 1C). All Clever-1 positive macrophages were macrophage mannose receptor (MRC) positive (a prototype marker for type 2 macrophages), but only about 50% of MRC-positive macrophages co-expressed Clever-1. Tumor cells induced Clever-1 expression in macrophages, since Clever-1 positive macrophages were not found in enlarged draining lymph nodes after a 17 d 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, 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 to 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 to 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 to the size of tumors in Clever^{-/-} mice (see Fig. 3A for comparison). If the size of tumors in ENDOclever^{-/-} mice were compared to 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 \textit{in vivo} both on macrophages and vascular endothelium. Moreover, since 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 lymph nodes.

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 interfere 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 lymph nodes (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 Figs. 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 to the control-treated animals, when the treatment was started before the tumor cell injections (Figs. 4A and 4B, 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 lymph nodes 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 three 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 intra-tumoral accumulation of type 2 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 positive T cells or in CD31 positive 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 to 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 2 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 since the numbers of intratumoral CD3 and CD8 positive 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 intra-tumoral blood vessels (CD31 and PV-1 positive) and lymphatics (CD31 positive and PV-1 negative) were comparable after anti-Clever-1- and control antibody-treatments in wild-type 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).

Since 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 since after anti-Clever-1 and control mAb administration similar proportion of intratumoral type 2 macrophages in primary tumors co-expressed Clever-1 (51.7 ± 5.9%, n = 5 and 52.2 ± 6.4%, n = 4 of type 2 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 towards type 2 cells significantly better than the culture medium alone (Supplementary Figs. S5B and S5C). Notably, 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

Since 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 positive T cells isolated from non-treated 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 positive blood lymphocytes to the tumor vasculature of Clever-1-treated mice was reduced in comparison to 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 prior to 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 ENDOclever-/- 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 anti-tumor 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. pro-inflammatory CCL3, IL-1 and IL-6). In the non-hematopoetic 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 negative population, the expression of pro-apoptotic Smad7 was increased and the expression of the anti-apoptotic 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 $46.4 \pm 4.8$/high-power field (HPF) and $15.7 \pm 5.7$/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 obtained also 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 immuno-modulatory 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 tumor-infiltrating leukocytes, since 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 2 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 2 macrophages under the local influence of tumor microenvironment, although differentiation of local tissue-resident macrophages may also contribute (32, 33). The key role of tumor-dependent differentiation is also supported by our findings showing that monocytes and macrophages co-cultured with B16 melanoma cells polarize towards type 2 macrophages in vitro. 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 2 macrophages. Our in vivo results clearly showed that the intra-tumoral monocytes/macrophages need more than 3 days to differentiate to MRC positive type 2 macrophages, differentiation to Clever-1 positive ones takes even longer (more than 6 days), and
the anti-Clever-1 treatment does not interfere with these kinetics (data not shown). The relatively late induction of Clever-1 in tumor macrophages has been found in other models as well (33). The anti-Clever-1 mAb did not deplete target antigen positive macrophages \textit{in vitro}. This is consistent with our earlier experiments demonstrating that \textit{in vivo} antibody treatment with anti-Clever-1 does not cause vascular damage or affect leukocyte counts (17). This is of course notwithstanding with the possibility that Clever-1 ligation might alter the functional responsiveness (e.g. immunosuppressive functions) of targeted macrophages.

Type 2 macrophages also induce regulatory T cells both via direct contacts (34) and via soluble mediators (32). For example, typical high IL-10 production of type 2 macrophages leads to the expansion of regulatory T cells, 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 2 macrophages. They in turn deliver suboptimal signals for maintaining normal numbers of regulatory T cells, which 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 i.e. 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 \textit{in vitro} assays supports this interpretation. It was technically not possible to
formally verify this hypothesis in vivo, since leukocyte accumulation in the tumor is slow when compared to lymph nodes 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 \textit{in vitro} assays (15). The numbers of Tregs and type 2 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 lymph nodes. 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 lymph nodes (17). Here we used an independent model for analyzing migration of subcutaneously injected cancer cells via the afferent lymphatics to draining lymph nodes 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 tumor infiltrating leukocytes. The inhibitory effects of anti-Clever-1 antibody therapy on progression of primary and metastatic tumors suggest that this molecule may be a new immune modulatory target for cancer immunotherapy. Since anti-Clever-1 treatment, unlike e.g. CTLA-4 inhibitors (36), did not aggravate autoimmune reactions, and since
it has a unique mode of action, it may have potential benefits in comparison to currently available immune modulating drugs.
Author contributions

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References


**Figure Legends**

**Figure 1.** Clever-1 is induced on tumor vasculature and a subset of tumor macrophages. A, two-color immunofluorescence staining of Clever-1 (green) on lymph nodes with anti-LYVE-1 (red) or anti-PV-1 (red) of WT mice. Thick arrows point to lymphatic vessels and a thin arrow points to a high endothelial venule and an arrowhead to a PV-1 positive flat walled venule. B, two-color staining of Clever-1 with biotinylated 1.26 antibody (red) and PV-1 with Meca-32 antibody (green) identifies the tumor vessels. Vascular positivity was confirmed with another monoclonal antibody (9-11) against the N-terminal 3 Kb fragment of Clever-1. The vessels are indicated by thin arrows and some macrophages (positive for Clever-1) by open arrows. C, immunofluorescence staining of melanoma metastases. Double staining with anti-macrophage mannose receptor, MRC (green) and anti-Clever-1 (red). Bars 100 μm in (A,B) and 20 μm in (C).

**Figure 2.** Generation and endothelial phenotype of different Clever-1 knockout mice. A, schematic presentation of the generation of different Clever-1 deficient mice. For verification of Clever-1/Stab-1 targeting (5’ end screening), DNA was digested with BamHI and hybridized with probe A (solid black line). Southern blot analysis of genomic DNA isolated from wild-type (Clever-1+/+) and from heterozygous (Clever-1+/-NEO+/-) ES cell clones. B, immunofluorescence stainings of tumors of Clever-/- mice showing the lack of Clever-1 on PV-1 positive (red) vessels. C, immunofluorescence staining of tumors of MACROclever-1-/- showing Clever-1 (green) positivity on PV-1 positive (red) blood vessels (thin arrows). D, immunofluorescence staining of ENDOclever-/- mice showing Clever-1 (green) positivity only on macrophages (open arrow) and LYVE-1 (red) positive lymphatics (arrow) in tumors. Staining with a negative control antibody is shown in the insets. Bars 100 μm.
Figure 3. Clever-1 deficiency leads to retarded tumor growth. A, combined results of three independent experiments demonstrating the size of primary tumors as relative luciferase counts ± SEM of B16 melanoma in WT (n = 18), full Clever-1-/- (n = 11) and MACROclever-/- (n = 21) mice at the end of the experiments (day 14). In the inset the raw values of one experiment are shown as an example. B, relative size of B16 melanoma in WT (n = 6) and ENDOclever-/- (n = 9) mice at the end of the experiment (day 14). Kinetics of the tumor development is shown in the inset in raw values. C, combined results of all experiments demonstrating the size of metastases of B16 melanomas in different genotypes. The metastases have been scored at the end of the experiments as explained in the Materials and Methods section. D, lymphoma cell migration in the rabbit. Fluorescently-labeled KCA lymphoma cells were subcutaneously injected into the footpads of rabbits treated with either anti-Clever-1 (n = 8) or control antibody (n = 9), and their appearance in the draining lymph nodes was detected by flow cytometry. The results are presented as relative migration (mean ± SEM). Migration after control antibody treatment is 1.0 by definition.

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 three 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).

**Figure 5.** Clever-1 deficiency and antibody therapy causes reduction in type 2 macrophages and FoxP3 positive lymphocytes in B16 melanoma. The results are presented as the mean number of positive cells ± SEM/high-power field (HPF). A, number of different cell populations in primary tumors and metastases of WT and Clever\(^{-/-}\) (KO) mice. Numbers of F4/80 positive macrophages, MRC positive macrophages, Foxp3 positive lymphocytes, CD3 positive T cells, CD8 positive T cells and CD31 positive vessels are shown as indicated. B, number of different cell populations in primary tumors and metastases in anti-Clever-1-treated and control antibody-treated mice. Numbers of FoxP3 positive lymphocytes, MRC positive macrophages, CD3 positive T cells, CD8 positive T cells, CD31 positive vessels with examples of immunofluorescence staining of primary tumors and metastases using anti-CD31 antibody in anti-Clever-1-treated and control antibody-treated mice and number of PV-1 positive vessels with examples of
immunofluorescence staining of primary tumors and metastases using anti-Meca-32 antibody (against PV-1) in anti-Clever-1-treated and control antibody-treated mice are shown as indicated. (3-5 tumors/genotype/treatment analyzed; 5 fields/tumor counted for all others except >30 fields/tumor for FoxP3 positive cells). Bar 100 μm.

**Figure 6.** 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 positive 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 pre-incubated 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 blockage 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, D, mRNA expression of the indicated target genes in CD45+ tumor-infiltrating leukocytes (C) and non-hematopoietic CD45− cells (D) 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 > 2 or < 0.6 are depicted. \( n = 6 \) mice analyzed in both groups.
Figure 3

Panel A shows the relative luciferase bioluminescence over time (D) for WT, Clever-1\(^{+/+}\), MACROclever\(^{-/-}\), and ENDOclever\(^{-/-}\) groups. The bar graph at Day 14 indicates a significant difference between the groups, with a p-value of 0.03.

Panel B displays the same data but emphasizes the trend over time. The line graph shows a consistent increase in luciferase bioluminescence for all groups, with WT having the highest activity.

Panel C illustrates the metastasis score for Control and α-Clever groups. The bar graph at Day 14 shows a significant reduction in metastasis score for the α-Clever group compared to Control, with a p-value of 0.03 for the first group and 0.015 for the second group.

Panel D presents the relative migration graph for Control and α-Clever groups. The bar graph indicates a significant decrease in relative migration for the α-Clever group, with a p-value less than 0.005.

Overall, these results suggest that Clever-1\(^{+/+}\) and MACROclever\(^{-/-}\) have a positive effect on luciferase bioluminescence and metastasis, while α-Clever has a negative effect on migration.
Figure 4

A

Relative luciferase bioluminescence

Day 10     Day 14
Primary tumor

P = 0.01   P < 0.01

Day 14
Metastasis

Control - Clever

B

Control                              α-Clever

Day 14
Metastasis

P < 0.01

C

Relative volume of the tumor

Day 7     Day 11     Day 14
Primary tumor

P < 0.01   P < 0.01

Day 14
Metastasis

P < 0.05   P < 0.01

D

Relative luciferase bioluminescence

Day 14     Day 20
Primary tumor

P < 0.0001

Day 14     Day 20
Metastasis

P < 0.0001
Figure 6

A and B: Graphs showing the percentage of control binding for Myeloid, Lymphocytes, and CD4+ cells. The x-axis represents different types of cells (TIL, BLOOD), and the y-axis represents the percentage of control binding. The graphs show significant differences with p-values of <0.0001.

C: Graph showing the expression of CD45+ cells with different RQ values.

D: Graph showing the expression of Non-hematopoietic cells with different RQ values.
# Clinical Cancer Research

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

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