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ORIGINAL RESEARCH

Expression of Tumor-mediated CD137 ligand in human colon cancer indicates dual signaling effects

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ABSTRACT

CD137-targeting immune therapy, which activates anti-tumor T effector cell responses, seems to be an attractive concept in clinical oncology. Recent evidence has demonstrated that tumor cells besides T cells and antigen-presenting cells are able to express CD137 and CD137L. Here we aimed to identify CD137/CD137L expression in established colon cancer cell lines and primary tumors (UICC stages I-IV) from patients with documented long-term follow-up. CD137/CD137L expression was highly upregulated in early to late-stage tumors while the inverse was observed in patient-derived peripheral blood mononuclear cells. High CD137L expression within primary tumors was mediated by tumor cells and significantly correlated with the occurrence of distant metastases and shortened survival in advanced stages of disease (UICC stage IV). Interestingly, induced tumor cell signaling via CD137L on its surface *in vitro* resulted in dual effects: (i) reduced tumor cell proliferation suggesting inhibitory signaling in all investigated cancers and (ii) increased epithelial-to-mesenchymal transition signaling events. Taken together CD137/CD137L expression was stage-dependently upregulated with shortened survival in patients with highly CD137L-expressing tumors. Our clinical and experimental data suggest that colon cancer cells predominantly express CD137L and thereby have negative impact on overall survival through a process of reverse signaling. Beside agonistic CD137 antibody therapy to foster T effector cell responses, CD137L-mediated intervention strategies may become instrumental to circumvent relapsed tumor growth through induced epithelial-to-mesenchymal transition and consecutive metastases formation.

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Introduction

Colorectal cancer (CRC) is a major cause of morbidity and mortality by solid tumors in industrialized countries. It accounts for almost 10% of all cancer incidences and is the third most common cancer and the fourth most common cause of death worldwide. CRC-related survival is highly dependent upon the stage of disease at the time of diagnosis and typically ranges from a 90% five-year survival rate for cancers detected at localized stages (T1-3 N0) to 50–70% for regional stages (T1-3 N1-2) and 25% for patients diagnosed with distant metastatic cancer (T1-4 N0-2 M1).[1]

CD137, also known as 4-1BB and Tumor Necrosis Factor (TNF) Receptor Superfamily member-9 (TNFRSF9), is a transmembrane glycoprotein of the TNF receptor superfamily and is involved in the development and activation of immune cells.[2] It is expressed on various populations of activated T cells including CD4+ and CD8+ T cells and binds with high affinity to the CD137 ligand (CD137L, also known as 4-1BB-L and TNFSF9).[3,4] In addition to the cell surface isoform of CD137, an alternatively spliced soluble isoform lacking the transmembrane domain exists which may be of relevance for therapeutic considerations. According to recent data, release of soluble CD137 (sCD137) is induced by malignant cells upon exposure to hypoxia as an immune escape mechanism to block CD137L-mediated T cell co-stimulation, effecting anti-tumor immune responses.[5]

Besides other decisive costimulatory ligands, CD137L is expressed on antigen-presenting cells (APCs) and myeloid progenitor cells. Under physiological conditions, CD137/CD137L interaction co-stimulates activation, proliferation, and/or survival of CD137 expressing cells, thus enhancing, for instance, CD8+ T cell-mediated anti-tumor immunity.[4,6] However, it can also promote the activation-induced cell death of repeatedly stimulated T cells.[4] Reverse signaling through CD137L inhibits the development of dendritic cells, B cells, and osteoclasts but supports mature dendritic cell survival and co-stimulates the proliferation and activation of mast cells.[7,8] Interestingly, CD137L expression has been detected on various tumor cells including non-small cell lung

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cancer (NSCLC) and CRC.[9,10] While signaling in immune cells showed both inhibiting and activating effects, the impact of CD137L induction on tumor cells is poorly understood. Recent data showed inhibition of tumor cell proliferation and apoptosis induction in cells from NSCLC.[9] In CRC, the role of tumor cell-related CD137/CD137L expression and its relevance in tumor progression have not yet been described.

To better understand the impact of CD137/CD137L in CRC, we specifically elucidated the influence of colon cancer cell-mediated CD137L expression on intracellular signaling and proliferation. In addition, we evaluated CD137 and CD137L expression in primary tumor tissues and peripheral blood mononuclear cells (PBMC) of patients with CRC at different stages of disease and analyzed the correlation of stage-dependent CD137/CD137L expression with clinicopathological characteristics and overall survival from long-term follow-up data.

Results

CD137 and CD137L are expressed by human colon cancer cells

CD137 and CD137L expression in primary colon cancers was first analyzed by immunofluorescence double-staining. Staining of CD137 or CD137L in combination with the epithelial cell adhesion molecule (EpCAM) marker clearly indicated stage-dependent expression of CD137 and CD137L on tumor cells of primary tumor tissue (Figure 1a,b, Table 1). In addition, CD137 and CD137L expression in tumor tissues of UICC stages II-IV was associated with CD4+ and CD8+ tumor infiltrated cells (Figure 2a and Table 1).

To confirm tumor cell-mediated expression of CD137 and CD137L in colon cancer, four established human colon cancer cell lines (HCT-116, HT-29, SW480, and SW620) were analyzed by flow cytometry and Western blot. In Western blot analysis, both CD137 and CD137L protein expression was detected in all investigated cell lines (Figure 2d). By flow cytometry, CD137L surface expression was detected in 24.5% (HT-29), 53.4% (HCT-116), 59.4% (SW480), and 73.9% (SW620) of cells (Figure 2b). Interestingly, when examining the proportion of CD137 positive cells no surface expression was detectable in all four cell lines indicating exclusive intracellular expression of CD137 in colon cancer cells. This was confirmed by intracellular staining (Figure 2c).

Upregulated CD137/CD137L colon cancer expression is associated with disease progression and metastases

CD137 and CD137L expression was analyzed by RT-qPCR in primary colon cancers and PBMCs from the colon cancer patients. In general, CD137 and CD137L expression was upregulated in all investigated colon cancers compared to normal colon tissues. Most colon cancer patients also demonstrated an increased expression in their PBMCs compared to healthy controls (CD137L: n = 72/76, 94.7% CD137: n = 72/81, 88.9%). For both CD137 and CD137L a stage-dependent effect was shown with further upregulated gene expression in the tumor corresponding with disease progression (CD137L: UICC I, fold difference (FD) = 12 vs. UICC II, FD = 40 vs. UICC III, FD = 62 vs. UICC IV, FD = 85; CD137: UICC I, FD = 30 vs. UICC II, FD = 48 vs. UICC III, FD = 74 vs. UICC IV, FD = 92; Figure 3a). In sharp contrast, expression in PBMCs was detected most intensely in early-stage patients while disease progression was associated with decreased expression profiles for both CD137 and CD137L (CD137L: UICC I, FD = 51 vs. UICC II, FD = 9 vs. UICC III, FD = 3 vs. UICC IV, FD = 3; CD137: UICC I, FD = 9 vs. UICC II, FD = 3 vs. UICC III, FD = 2 vs. UICC IV, FD = 2; Figure 3b).

To examine whether the expression of CD137 corresponded with CD137L in the tumor or PBMCs, regression analysis was performed. In tumors and PBMCs CD137 expression demonstrated a significant direct correlation with the expression of CD137L (primary colon cancers: $R^2 = 0.25$, $p < .001$; PBMC: $R^2 = 0.64$, $p < .0001$; Figure 3c).

Significant differences in the distribution between high and low CD137 expression were observed regarding UICC stage and T category (both $p<0.05$) while CD137L expression varied significantly regarding UICC stage ($p<0.005$), lymph node metastases ($p<0.0001$), and distant metastases ($p<0.05$) (Table 2). Significant differences for both CD137 and CD137L expression were observed regarding UICC stage (both $p<0.05$) and distant metastases (both $p<0.05$) (Table 3).

High CD137L expression in primary tumors (FD > 17) was significantly correlated with the occurrence of distant metastases (UICC stage IV) and shortened survival in advanced stage of disease when compared to CD137L low expression (FD ≤ 17; Figure 4a). This difference was not observed for high CD137 expression (FD > 30; Figure 4b). High CD137L expression showed a significant correlation with worsened overall survival in UICC stage III and UICC stages III + IV but no significant correlation was observed for high CD137 expression (Figure 4c,d). Interestingly, analysis of CD137 + CD137L expression showed a significant correlation for high expression and worsened overall survival in UICC stage III but not in UICC stage IV in which patients had distant metastases and thus high tumor burden (Figure 4e).

Activation of isolated colon cancer cells via CD137 ligand resulted in reduced tumor cell proliferation

To dissect the effects of tumor cell-mediated CD137L signaling on tumor cell proliferation, MTS proliferation assays were performed. When compared to untreated control cells activation through CD137L on the surface of colon cancer cells by immobilized CD137-Fc resulted in significantly reduced tumor cell viability in all investigated cell lines (HT-29: 87%; HCT-116: 87% (both $p<0.05$)) and SW480: 84%; SW620: 78% (both $p<0.005$) (Figure 5a). Interestingly, Luminex analysis demonstrated sCD137 in supernatants of cancer cells exposed to CD137-Fc whereas no sCD137 was detected in supernatants from untreated control cells indicating a negative feedback loop for survival of the cancer cells (HT-29, 9ng/ml, HCT-116, 12 ng/ml, SW480, 16 ng/ml, SW620, 18 ng/ml; Figure 5b).

In addition, induction of CD137L caused significantly elevated release of both growth factors VEGF and PDGF (VEGF: HT-29, 137%, $p<0.05$; HCT-116, 144%, $p<0.0001$; SW480, 113%, $p<0.005$; SW620, 111%, $p<0.05$, and PDGF: HT-29,

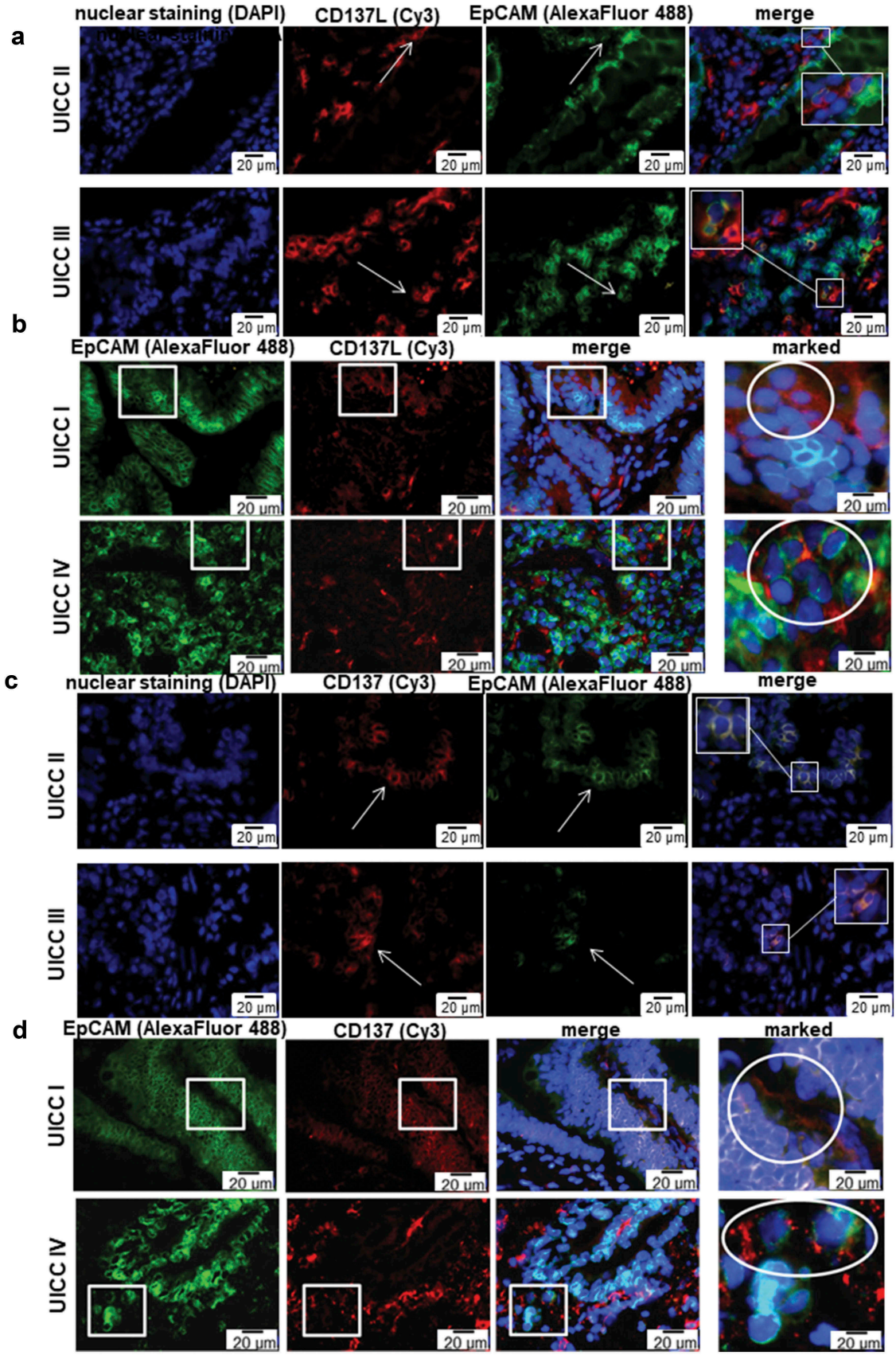

Figure 1. Representative examples of immunofluorescence co-staining for CD137L and CD137 (red) and EpCAM (green) of tissues from patients with UICC stage I-IV colon cancer. (a) CD137L and (b) CD137 expressing tumor cells in human colon cancer tissues. AlexaFluor 488, green; Cy3 (indocarbocyanin), red; DAPI (49,6-diamidino-2-phenylindoldihydrochlorid), blue – nuclear counterstaining.

Table 1. Immunofluorescence tumor tissue analysis from UICC stage I-IV CRC patients.

	UICC I	UICC II	UICC III	UICC IV
CD137 Ligand/EPCAM	+	+/++	+++	++/+++
CD137/EPCAM	0/+	+	++	++
CD137/CD4	+	++	+/++	+
CD137/CD8	+	++	++	+
CD137/CD4	0/+	+	+	+
CD137/CD8	0	0/+	+	0/+

Semiquantitative interpretation of tumor tissue sections of two independent investigators (0: no positivity; 0/+: faint, +: minor; ++: moderate; +++: distinct).

132%, $p<0.005$; HCT-116, 123%, $p<0.05$; SW480, 124%, $p<0.005$; SW620, 120%, $p<0.05$; Figure 6).

To further clarify the observed effects we performed Western blot analysis to investigate the activation of the VEGF/PDGF-targeted MAPK and PI3K/Akt/mTOR pathways. Interestingly, phosphorylation of both the PI3K/Akt/mTOR pathway component Akt and the MAPK pathway component Erk was found to be decreased after induction of CD137L by immobilized CD137-Fc (pAkt: HT-29, relative optical density (ROD) = 58%; HCT-116, ROD = 51%; SW480, ROD = 62%; SW620, ROD = 67% and pErk: HT-29, ROD = 75%; HCT-116, ROD = 77%; SW480, ROD = 53%; SW620, ROD = 52%; Figure 7A) indicating that neither VEGF nor PDGF exerts its function via autoregulative self-induction of their receptors (VEGF and PDGFR).

CD137L induction causes elevated expression of Vimentin and TLR7

Since high CD137 and CD137L expression was associated with advanced tumor stages and thus the occurrence of distant metastases in our patient cohort, we analyzed the expression of Vimentin, an intermediate filament protein often used as a marker for cells undergoing epithelial-to-mesenchymal transition (EMT).[11] Interestingly, Western blot analysis demonstrated increased expression of Vimentin in colon cancer cells after CD137-Fc induced CD137L signaling (HT-29: ROD = 148%, HCT-116: ROD = 136%, SW480: ROD = 349%, and, SW620: ROD = 292% cells; Figure 7b).

Previously we demonstrated association of Toll-like receptor (TLR) 7 and TLR8 expression with tumor-initiating cells in human colon cancer.[12] While no differences in TLR8 expression were identified after CD137L induction (data not shown), TLR7 expression was detected to be profoundly increased after CD137L engagement in three out of four cell lines (HCT-116: ROD = 630%, SW480: ROD = 548%, and SW620: ROD = 382%; Figure 7b).

Since TLR stimulation has been found to be associated with increased tumor cell proliferation in inflammation-linked tumors like pancreatic cancer, we performed proliferation assays using the TLR7 (and TLR8) agonist Resiquimod (R848).[13,14] Cells cultured in un-coated and CD137-Fc pre-coated plates demonstrated significantly elevated tumor cell proliferation after treatment with R848, while a further increase was observed in pre-coated plates compared to uncoated controls (HT-29: 112% vs. 121%, HCT-116: 113% vs. 121%, SW480: 120% vs. 128%, SW620: 117% vs. 132%; Figure 8).

Discussion

The presented results provide evidence for the first time of an increased tumor-mediated expression of CD137 and its corresponding ligand in colon cancer that is associated with adverse prognosis, particularly in advanced UICC stage IV patients with the occurrence of distant metastases. In primary colon cancers significantly increased expression of CD137 and CD137L was detected. Moreover, analysis of distribution frequency demonstrated high CD137/CD137L tumor-mediated expression to be associated with metastases formation in our patient cohort. Out of 28 colon cancer patients with diagnosed distant metastases at the time of surgery, 68% (19/28) and 71% (20/28) showed high CD137 or CD137L expression within their tumors which was positively correlated with shortened overall survival for CD137L (Figure 4). Tumor cell-mediated CD137 and CD137L expression was confirmed *in vitro* by analysis of four established human colon cancer cell lines all expressing CD137 and its ligand. In previous studies, tumor-associated CD137 and CD137L expression was demonstrated in several human primary tumors such as colon, pancreatic, lung, and breast cancer. Both CD137 and the corresponding ligand were detected in the tumor tissues of the analyzed patients, but not the corresponding normal tissues from these individuals. While CD137L was expressed on the tumor cells, CD137 was instead found on the vessel walls within the tumors. In addition, these studies demonstrated the expression of CD137 and CD137L more commonly in malignant tumors with moderate or poor differentiation than compared to well-differentiated tumors.[15,16] Interestingly, while our Western blot analysis confirmed CD137 expression in all investigated cell lines, our flow cytometry results showed no CD137 cell expression. Immunofluorescence double-staining also demonstrated expression of CD137 that was suggested to be more pronounced intracellularly rather than on the surface of the tumor cells. Taken together these results indicate almost exclusive intracellular expression in colon cancer cells. Moreover, recently published data showed release of an alternatively spliced soluble form of CD137 (sCD137) upon exposure to hypoxia in murine cell lines from colon and renal cell carcinoma, thymoma, and melanoma.[5] The authors demonstrated that sCD137 released by tumor cells inhibited CD137L-mediated T cell co-stimulation thus representing an immune escape mechanism of the tumor. Interestingly, our Luminex analysis confirmed release of sCD137 by all four investigated colon cancer cell lines upon CD137L activation, suggesting internalized CD137 expression in the colon cancer cells that can be released from vesicles upon stimulation for immune escape.

More interestingly, our functional analysis using CD137-Fc demonstrated significantly inhibited tumor cell proliferation via CD137L expression on the tumor cell surface. Recent data also points to decreased proliferation upon CD137L reverse signaling in hepatocellular carcinoma and non-small cell lung cancer[9,17] Our results were puzzling at first as expression analysis in primary colon cancers is generally linked to high CD137L expression in the tumor and poor prognosis, particularly in UICC stage III and IV

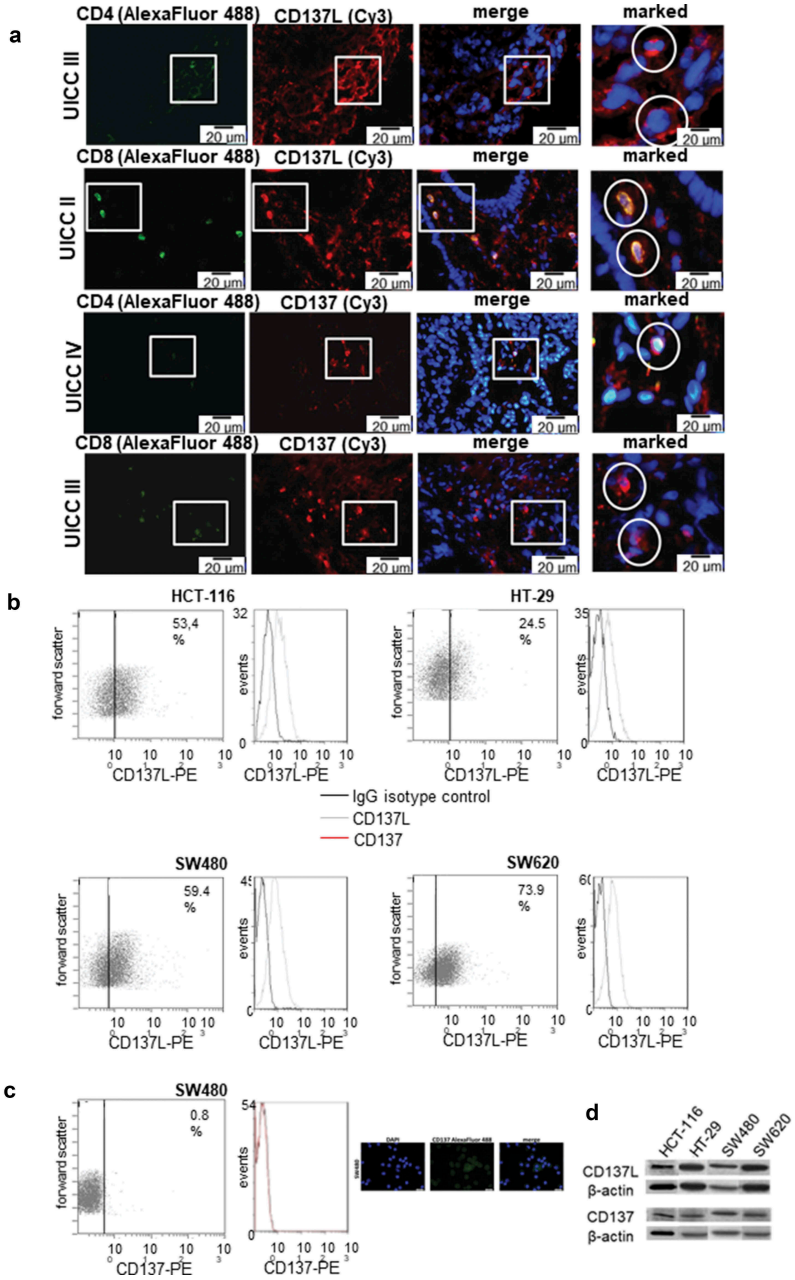

Figure 2. (a) Representative examples of immunofluorescence co-staining for CD4 and CD8 (green) and CD137L and CD137 (red) of tissues from patients with UICC stage II-IV colon cancer. (b) Flow cytometric analysis of HCT-116, HT-29, SW480, and SW620 colon cancer cells using PE-conjugated antibodies against CD137L. (c) Flow cytometric analysis (SW480, representative example for negative surface staining in all four investigated cell lines) and immunofluorescence (SW480, representative example for positive intracellular staining in all four investigated cell lines) for CD137. FACS analysis: CD137L depicted as grey line, CD137 as red line, isotype control as black line, PE (phycoerythrin); immunofluorescence: DAPI (49,6-diamidino-2-phenylindoldihydrochlorid), blue – nuclear counterstaining, AlexaFluor 488, green. (d) Western blot analysis of CD137L and CD137 expression in human colon cancer cell lines. β-actin probe was used as a control for protein loading.

cancer patients with local and distant metastases formation. CD137L stimulation did not only result in reduced tumor cell proliferation but at the same time caused elevated expression of Vimentin and TLR7. Vimentin is an intermediate filament protein that is upregulated in cells undergoing epithelial-to-mesenchymal transition (EMT) and therefore is often used as a marker for this process.[11] EMT is required to allow individual cells of the tumor to detach from primary sites, enter the bloodstream, and eventually establish metastases in a reversed process termed mesenchymal-to-epithelial transition (MET).[18] Our clinical and experimental results reveal, for the first time, a potential CD137L stimulatory effect on EMT and metastasis formation of colon cancer cells. Previous work using a murine colon cancer model showed that CD137L signaling suppressed intratumoral differentiation of IL-12-producing CD103+ dendritic cells (DCs) and type 1 tumor-associated macrophages (TAMs), both of which are crucial for generating IFNγ-producing CD8+ T cells,[8] suggesting that CD137L signaling functions as a negative regulator of anti-tumor immunity. As such, therapy-induced blockade of the CD137L signaling pathway may be used not

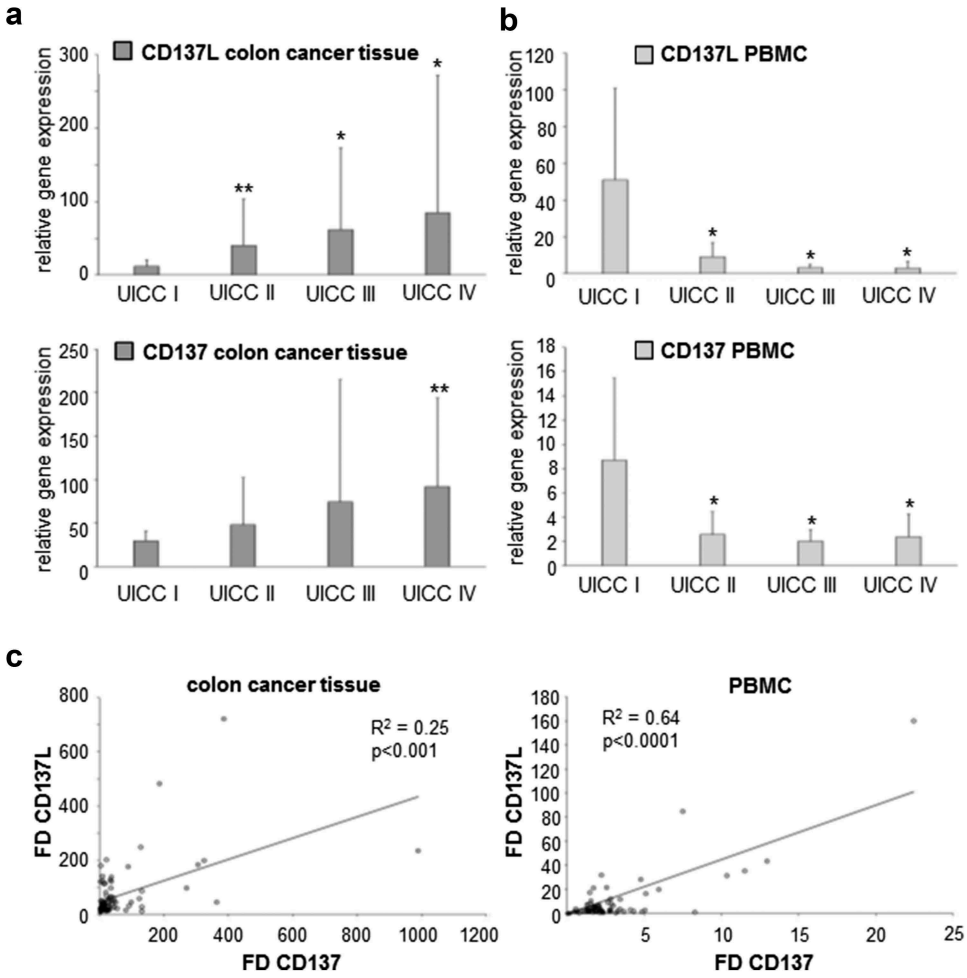

Figure 3. (a) Increased CD137L or CD137 gene expression in late-stage colon cancer tissue compared to early stages. Values for normal colon tissue were standardized to baseline. The relative gene expression is expressed as $2^{-\Delta\Delta Cq}$ (fold difference, FD). (b) Decreased CD137L or CD137 gene expression in PBMCs (peripheral blood mononuclear cells) of patients with late-stage colon cancer compared to early stages. Values from eight healthy control probands were standardized to baseline. The relative gene expression is expressed as $2^{-\Delta\Delta Cq}$ (FD). (c) Significant correlation of CD137 with CD137L relative gene expression (FD) in colon cancer tissue and PBMCs. Pearson's correlation coefficient was used to describe and test bivariate correlations. R2, coefficient of determination. *p < .05, **p < .005.

only to inhibit EMT and metastasis formation but also to promote immunosurveillance through induction of differentiation of IL-12-producing CD103+ DCs/type 1 TAMs and thereby activation of anti-tumoral IFNγ-producing cytotoxic CD8+ T cells. TLRs are a family of receptors that are associated with the maintenance of tissue homeostasis via the regulation of inflammatory and tissue repair responses to injury.[19] Recently, enhanced expression of TLRs has been described in a variety of solid tumors including colon, pancreatic, and breast cancer.[12,13,20–22] A study analyzing TLR3, TLR4, and TLR9 in breast cancer associated TLR expression with the risk for metastases.[20] In addition, our previous studies showed that TLR7 and TLR8 were co-expressed with CD133, a marker for cancer-initiating cells in CRC.[12] The underlying theory of cancer-initiating cells assumes that cancer tissues contain a minor population of cells that is predominantly or exclusively associated with tumor growth and metastases formation, and is additionally characterized by drug resistance.[23] In accordance with this, we demonstrated previously that TLR7 induction was associated with increased tumor growth and chemotherapeutic resistance in pancreatic cancer.[13] Here, we demonstrated elevated TLR7 expression upon CD137L activation in colon cancer cells, putatively a reverse feedback loop for tumor growth reactivation in such cancer-initiating cells. In addition, TLR7 stimulation resulted in increased tumor cell proliferation which was further increased in tumor cells exposed to immobilized CD137-Fc.

Moreover, CD137L stimulation in colon cancer cells also leads to the enhanced release of the growth factors VEGF and PDGF. In previous studies, we demonstrated that treatment with VEGF and PDGF resulted in increased tumor cell proliferation of colon cancer.[24] Additionally, we hypothesized that tumor-mediated VEGF and PDGF expression may lead to an autoregulative self-induction of VEGF and PDGF receptors on pancreatic cancer cells.[14] To exclude autoregulative effects of VEGF and PDGF in our experimental setting, we performed Western blot analyses to investigate VEGF/PDGF targeted MAPK and PI3K/Akt/mTOR pathway components pAkt and pErk. Phosphorylation of both Akt and Erk was found to decrease after induction of CD137L. Consequently, as VEGF and PDGF are also known to be involved in tumor cell migration and angiogenesis, we believe that the observed increase in extracellular VEGF and PDGF expression together

Table 2. Distribution of PBMC gene expression by demographic and disease characteristics.

	CD137			CD137L		
	low	high		low	high	
Gender						
male	21 (45%)	26 (55%)		23 (55%)	19 (45%)	
female	16 (47%)	18 (53%)		13 (39%)	20 (61%)	
Age (y)						
≤ 65 years	15 (39%)	23 (61%)		16 (50%)	16 (50%)	
≤ 65 years	22 (51%)	21 (49%)		20 (47%)	23 (53%)	
> 65 years						
UICC stage						
I	0 (0 %)	8 (100%)	$p < .05$	0 (0 %)	8 (100%)	$p < .005$
II	10 (45%)	12 (55%)		6 (29%)	15 (71%)	
III	13 (57%)	10 (43%)		12 (63%)	7 (37%)	
IV	14 (50%)	14 (50%)		18 (67%)	8 (33%)	
Depth of Invasion						
pT2	3 (19%)	13 (81%)	$p < .05$	4 (29%)	10 (71%)	n.s.
pT3	25 (54%)	21 (46%)		23 (51%)	22 (48%)	
pT4	9 (47%)	10 (53%)		9 (56%)	7 (44%)	
Lymph node metastases						
N0	15 (42%)	21 (58%)	n.s.	8 (23%)	27 (77%)	$p < .0001$
N1	13 (57%)	19 (43%)		16 (73%)	6 (27%)	
N2	9 (41%)	13 (59%)		12 (67%)	6 (33%)	
Distant metastases						
M0	23 (43%)	30 (57%)	n.s.	18 (37%)	30 (63%)	$p < .05$
M1	14 (51%)	13 (48%)		17 (65%)	9 (35%)	
Differentiation						
G2	26 (48%)	28 (52%)	n.s.	25 (49%)	26 (51%)	n.s.
G3	9 (45%)	11 (55%)		5 (29%)	12 (71%)	

Frequency distributions of CD137 and CD137L were compared using kxm tables (Chi-squared). Mean cut-off values for either high or low expression were set FD ≤ 2.0 for CD137 and FD ≤ 2.9 for CD137L. y, years; UICC, Union Internationale Contre le Cancer; n.s., not significant.

Table 3. Distribution of colon cancer tissue gene expression by demographics and disease characteristics.

	CD137			CD137L		
	low	high		low	high	
Gender						
male	29 (57%)	22 (43%)	n.s.	25 (49%)	26 (51%)	n.s.
female	12 (35%)	22 (65%)		15 (44%)	19 (56%)	
Age (years)						
≤ 65 years	15 (39%)	24 (62%)	n.s.	16 (41%)	23 (59%)	n.s.
> 65 years	26 (57%)	20 (43%)		24 (52%)	22 (48%)	
UICC stage						
I	5 (63%)	3 (38%)	$p < .05$	6 (75%)	2 (25%)	$p < .05$
II	16 (70%)	7 (30%)		10 (43%)	13 (57%)	
III	11 (44%)	14 (56%)		16 (64%)	9 (36%)	
IV	9 (31%)	20 (69%)		8 (28%)	21 (72%)	
Depth of Invasion						
pT2	8 (50%)	8 (50%)	n.s.	9 (56%)	7 (44%)	n.s.
pT3	25 (52%)	23 (48%)		25 (52%)	23 (48%)	
pT4	8 (38%)	13 (62%)		6 (29%)	15 (71%)	
Lymph node metastases						
N0	23 (62%)	14 (38%)	n.s.	19 (51%)	18 (49%)	n.s.
N1	10 (42%)	14 (58%)		11 (46%)	13 (54%)	
N2	8 (33%)	16 (67%)		10 (42%)	14 (58%)	
Distant metastases						
M0	32 (57%)	24 (43%)	$p < .05$	31 (55%)	25 (45%)	$p < .05$
M1	9 (32%)	19 (68%)		8 (29%)	20 (71%)	
Differentiation						
G2	32 (57%)	24 (43%)	n.s.	28 (50%)	28 (50%)	n.s.
G3	8 (36%)	14 (64%)		8 (36%)	15 (64%)	

Frequency distributions were compared using kxm tables (Chi-squared). Mean cut-off values for either high or low expression were set FD ≤ 30.0 for CD137 and FD ≤ 17.0 for CD137L. y, years; UICC, Union Internationale Contre le Cancer; n.s., not significant.

with upregulated Vimentin and TLR7 takes place to support metastatic processes in colon cancer upon induction of CD137L.[24,25]

Beyond the expression in the tumor, we also investigated CD137/CD137L profiles in PBMCs from each patient. Around 90% of the investigated colon cancer patients showed increased expression of both CD137 and CD137L compared to healthy controls, indicating activated immune responses in the tumor patients. Interestingly, we observed significant decrease of CD137 and CD137L expression in PBMCs from patients with

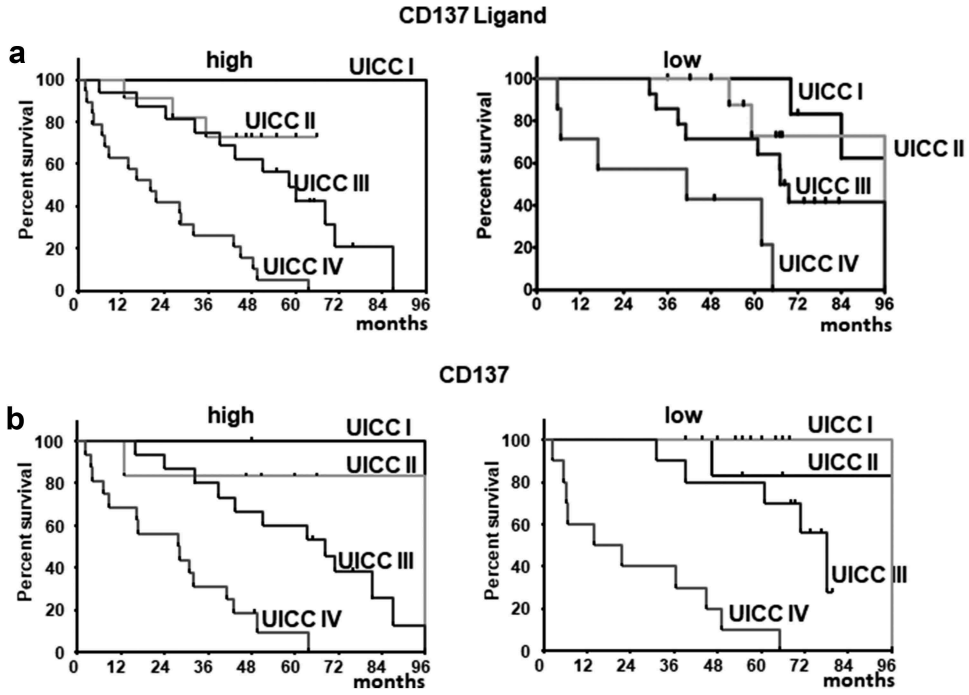

Figure 4. Overall survival of colon cancer patients of UICC stages I-IV with high versus low CD137 and CD137L gene expression in tumor tissues. (a) High CD137L expression was significantly correlated with the occurrence of distant metastases (UICC stage IV) and shortened survival in advanced stage of disease when compared to low CD137L expression. (b) This correlation was not observed for high versus low CD137 expression. Log-rank test showed a significant correlation for high CD137L expression and worsened overall survival in (c) UICC stage III and UICC stages III + IV and no significant correlation for high CD137 in (d) UICC stage III and UICC stages III + IV. (e) A composite of CD137 + CD137L expression showed a significant correlation for high expression and worsened overall survival in UICC stage III and no significant correlation in UICC stage IV. Mean cut-off values for low and high expression of CD137 and CD137L in tumor tissues were set as FD ≤ 30, FD > 30 and FD ≤ 17, FD > 17, respectively. $p < .05$ was considered statistically significant.

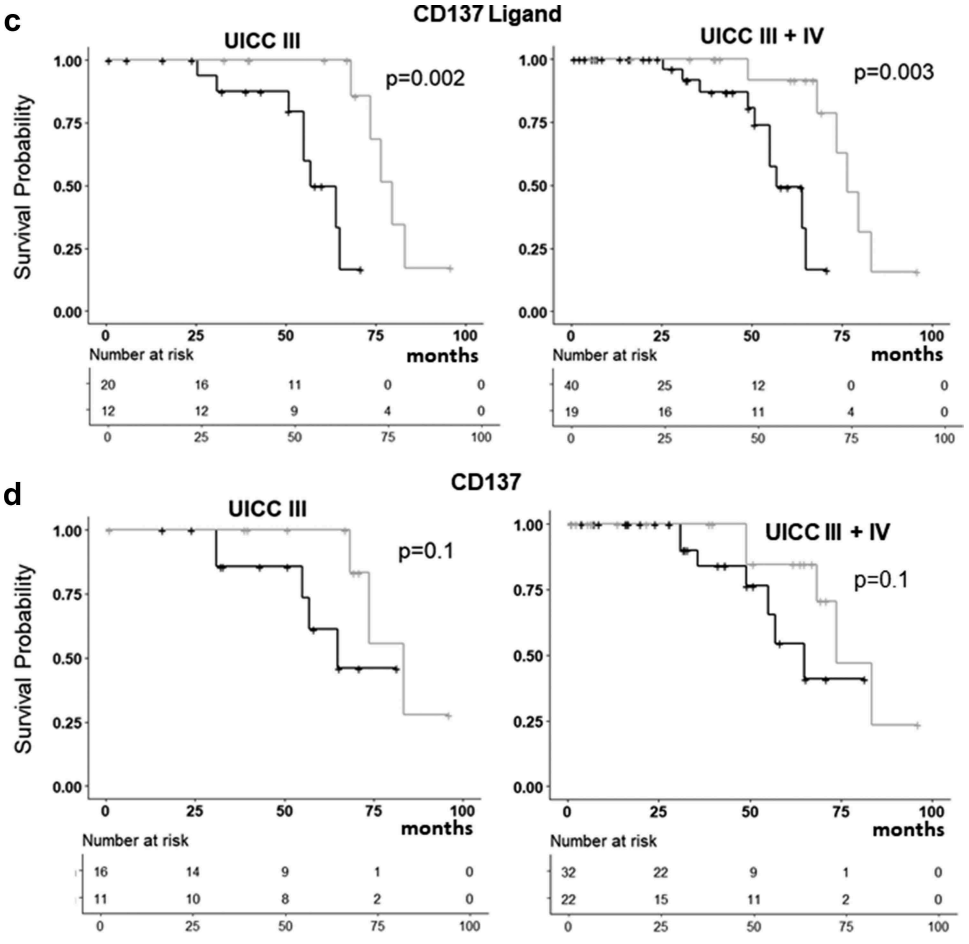

Figure 4. Continued.

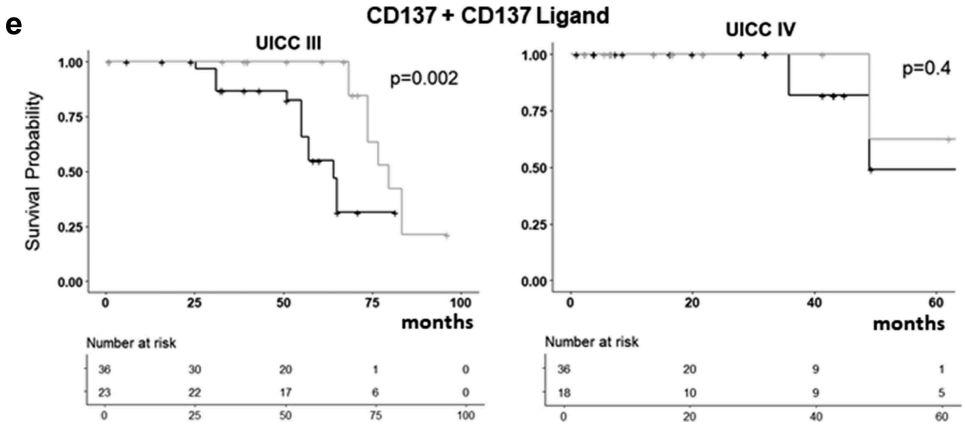

Figure 4. Continued.

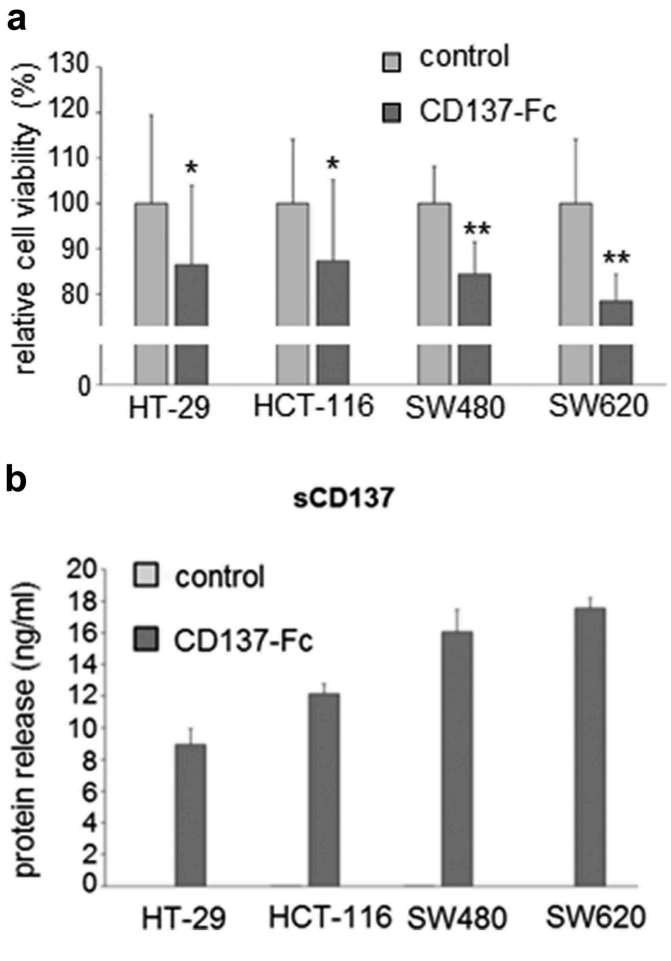

Figure 5. (a) MTS proliferation assay of colon cancer cell lines treated with CD137-Fc for CD137L activation. Relative cell viability of HT-29, HCT-116, SW480, and SW620 cells was analyzed 48 h after application. Untreated control cells were standardized to baseline. *p < .05, **p < .005. (b) Luminex analysis of soluble CD137 (sCD137) in supernatants of human colon cancer cells after treatment with CD137-Fc for CD137L induction. Supernatants were analyzed 48 h after application. *p < .05, **p < .005, ***p < .0001.

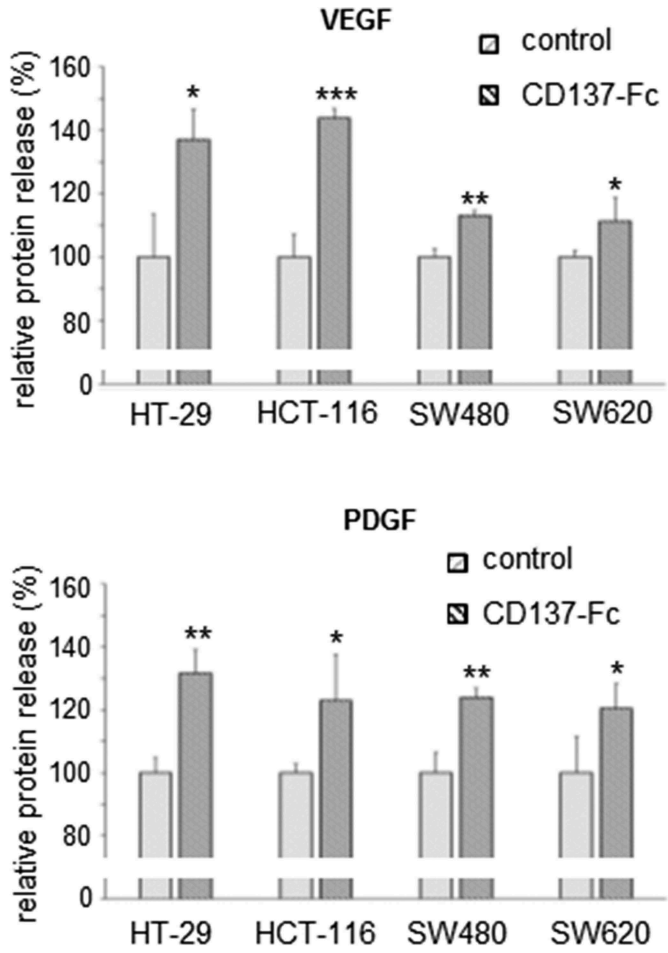

Figure 6. Luminex analysis of VEGF and PDGF in supernatants of human colon cancer cells after treatment with CD137-Fc for CD137L induction. Supernatants were analyzed 48 h after application. Untreated control cells were standardized to baseline for VEGF and PDGF measurements. *p < .05, **p < .005, ***p < .0001.

disease progression (UICC stage III and IV), suggesting functional immune exhaustion that may be particularly relevant in late-stage tumor patients with progressing tumor mass.

CD137 ligation with agonistic antibodies to particularly induce T cell co-stimulation and to foster effective anti-tumor immune responses was first used to treat murine tumors. As a monotherapy, CD137 monoclonal antibodies (mAb) were

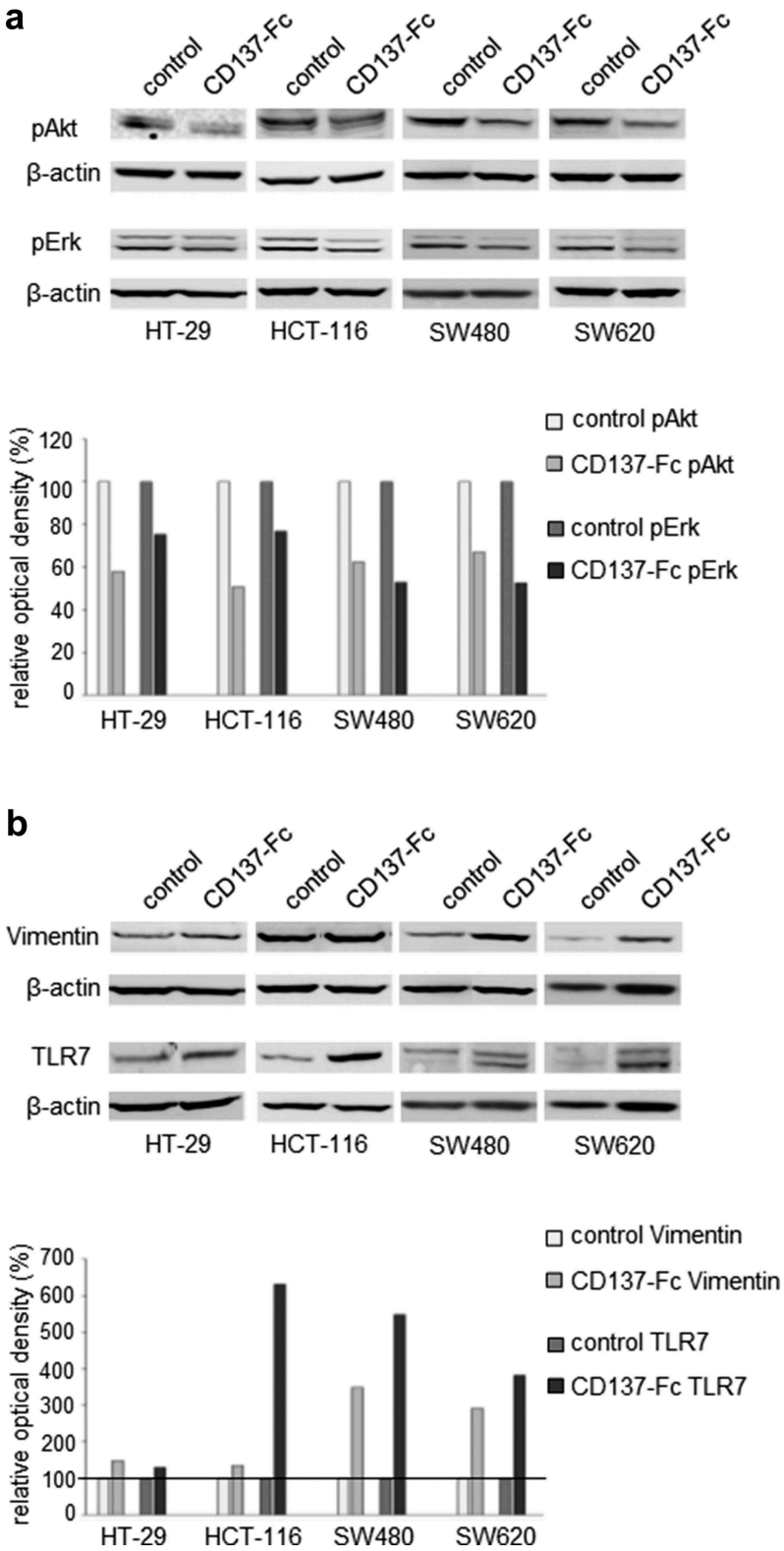

Figure 7. Western blot analysis of (a) pAkt and pErk and (b) Vimentin and TLR7 in human colon cancer cell lines after exposure to CD137-Fc for CD137L activation. ß-actin probe was used as a control for protein loading. Relative optical density (ROD) was determined using a Bio-Rad ChemiDoc Touch Imaging System with ImageLab Touch Software. Untreated control cells were standardized to baseline.

found to be capable of controlling tumor growth or promoting complete remission in a variety of grafted tumors including sarcomas, colon carcinomas, and lymphomas.[26,27] Clinical approaches instead focus on the promising aspects of anti-CD137 mAb therapy in combination with other immunotherapeutic treatments. Early clinical data from combinations of anti-PD-1 blocking antibodies with agonistic anti-CD137 antibodies or anti-CD137 antibodies as a monotherapy showed

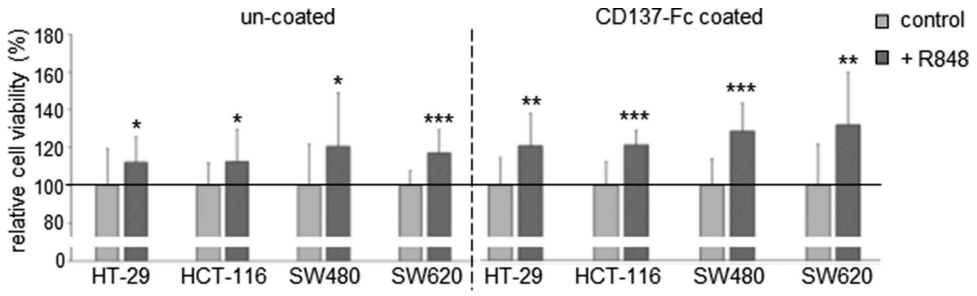

Figure 8. MTS proliferation assay of colon cancer cell lines treated with TLR 7 (and TLR8) agonist Resiquimod (R848) including (coated) or without (uncoated) exposure to CD137-Fc for CD137L activation. Relative cell viability of HT-29, HCT-116, SW480, and SW620 cells was analyzed 48 h after first application of R848. Untreated control cells were standardized to baseline.

Table 4. Patient demographics and disease characteristics.

	cases (n)	%
Gender		
male	52	60
female	24	40
Age		
≤ 65 years	40	47
> 65 years	46	53
UICC stage		
I	8	9
II	24	28
III	25	29
IV	29	34
Depth of Invasion		
pT2	16	19
pT3	49	57
pT4	21	24
Lymph node metastases		
N0	37	43
N1	24	28
N2	25	29
Distant metastases		
M0	57	67
M1	29	33
Differentiation		
G1	7	8
G2	57	66
G3	22	26

UICC, Union Internationale Contre le Cancer.

promising results in phase I/II trials.[28–31] However, efficacy data demonstrating a survival benefit for colorectal cancer remains to be seen.

In summary, we presented for the first time a detailed CD137/CD137L expression analysis together with long-term clinical outcome data and functional results from *in vitro* studies. These results support ongoing clinical trials of CD137 ligation with agonistic antibodies to induce CD8+ and NK cell activation and to foster anti-tumor immune responses in colorectal cancer. CD137/CD137L was found to be highly expressed in PBMCs and within the tumor, underlining the significance of this pathway particularly in CRC patients. Moreover, colon cancer cells by themselves, as shown in this work, are able to stage-dependently express CD137L on their surface particularly in advanced-stage tumors of patients with poor survival. In addition to this latter finding, we also demonstrated for the first time a suggestive dual effect of CD137 ligation. Reverse CD137 binding via its ligand on the surface of the tumor cells resulted in reduced colon cancer cell proliferation. Interestingly, reverse CD137L signaling induced intracellular epithelial-to-mesenchymal transition signaling events that may result in negative (i.e. pro-metastatic) effects in colon cancer and therefore demands further investigation.

Materials and methods

Patients, human tissue and peripheral blood mononuclear cells

Eighty-six patients with histologically confirmed colon cancer undergoing curative surgical resection in our department between 07/2002 and 10/2010 were included in the study. The study was conducted in accordance with the Declaration of Helsinki. Informed consent was obtained preoperatively, as was approved by the local medical ethics committee. The histological stage of the tumor was determined according to the *Union Internationale Contre le Cancer* (UICC) TNM staging system.[32] Tumors were evaluated for stage and differentiation grade. Data concerning age, gender, level of wall infiltration, lymph node metastasis, and distant metastases were collected in a database. Clinical characteristics of the study population are summarized in Table 4. For subsequent RNA extraction tumor biopsies were stored in RNAlater (Qiagen, Hilden, Germany) and immediately frozen in liquid nitrogen. For cryostat sections, samples were embedded in Tissue-Tek OCT compound (Sakura, Torrance, CA, USA). Serial cryostat sections (5 mm) were made using a Cryostat Leica CM3050 S and mounted on microscope glass slides. Peripheral blood mononuclear cells (PBMC) were obtained from whole blood samples by ficoll density gradient separation.

Cell culture and in vitro activation of CD137L

The four human colon cancer cell lines HT-29, HCT-116, SW480, and SW620 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). HT-29 and HCT-116 cells were cultured in McCoy Medium (ATCC) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin (pen/strep). SW480 and SW620 cells were maintained in RPMI 1640 medium (ATCC) containing 10% (v/v) FBS and 1% (v/v) pen/strep. All cell lines were incubated at 37°C in 5% CO_2. To induce CD137L signaling cell culture plates were coated with 10 µg/ml recombinant human CD137-Fc (R&D Systems, Minneapolis, MN, USA) at 4°C overnight. To preserve cell surface epitopes cells were then carefully

detached using accutase solution (Sigma-Aldrich, St. Louis, MO, USA) and seeded in the pre-coated wells in a defined number.

Immunofluorescence

Primary antibodies against CD137 and CD137L were purchased from abcam (Cambridge, UK), antibody to EpCAM was obtained from Cell Signaling Technology (Danvers, MA, USA). Secondary Cy3 (indocarbocyanin) conjugated and AlexaFluor 488 conjugated antibodies were obtained from Jackson ImmunoResearch (West Grove, PA, USA). The staining was performed on cryostat sections of snap-frozen tumor specimens and cytospin preparations of human colon cancer cell lines. Samples were fixed in acetone, permeabilized in methanol, and incubated with the primary antibody in TBS plus 0.5% bovine serum albumin (BSA) overnight at 4°C in a humidified chamber. Treatment with the secondary fluorochrome-conjugated antibody was performed for 30 min at room temperature in a humidified chamber. Subsequently, slides were mounted with DAPI Fluoromount-G (Southern Biotech, Birmingham, AL, USA) and analyzed using an Olympus BX51 microscope and the CellSens Dimension software. The immunofluorescence staining protocol was identical for tissue sections and cytospin samples. Two independent investigators analyzed tumor tissue sections and cytospin preparations of human colon cancer cell lines using a semiquantitative interpretation assessment (0: no positivity; 0/+: faint, +: minor; ++: moderate; +++: distinct). In the case of divergent results, mutual consent was obtained.

RNA extraction and quantitative real-time PCR (Rt-qPCR)

Gene expression of CD137 and CD137L in human colon cancer tissue and PBMCs was determined using quantitative real-time PCR (RT-qPCR). Total cellular RNA was extracted using RNeasy Minikit (Qiagen, Hilden, Germany) on the QIAcube platform (Qiagen) according to the manufacturer's instructions. Reverse transcription was performed using ImProm II reverse transcriptase system (Promega, Mannheim, Germany) and Eppendorf Mastercycler (Eppendorf, Hamburg, Germany). For analysis of colon tissue samples, human colon-matched cDNA was purchased from Pharmingen (Heidelberg, Germany) and used as a control. For analysis of patients' PBMCs, samples from eight healthy probands were used as a control. PCR was performed using Taqman Gene Expression Master Mix (Life Technologies, Carlsbad, CA) and Taqman Gene Expression Assay (Life Technologies) in concordance to the manufacturer´s instructions and carried out in duplicates on a Biorad CFX96 Touch Real-Time PCR Detection System. Housekeeping genes β-actin, B2M (β 2 microglobulin), and RPLP0 (ribosomal protein, large, P0) were used for relative quantification. Reproducibility was confirmed by three independent PCR runs. The relative gene expression value, fold difference (FD), is expressed as $2^{-\Delta\Delta Cq}$.

Flow cytometry (FACS)

Colon cancer cell lines HT-29, HCT-116, SW480, and SW620 were analyzed for the expression of CD137 and CD137L using a flow cytometer Coulter EPICS XL (Beckman Coulter, Krefeld, Germany) with a software package Coulter Epics XL-MCL System II (Beckman Coulter). Phycoerythrin (PE)-conjugated CD137 and CD137L antibodies as well as isotype control antibodies were purchased from Miltenyi (Bergisch Gladbach, Germany).

Western blot

Protein extraction from colon cancer cell lines was performed using lysis buffer RIPA containing DDT (Sigma-Aldrich, Steinheim, Germany) and protease/phosphatase inhibitor cocktails (Merck Millipore, Billerica, MA, USA). For preparation of cell lysates adherent cells were supplemented with lysis buffer and transferred to an eppendorf tube. Samples were incubated on a rotator at 4°C for 30 min, then centrifuged (full speed, 4°C, 20 min) and supernatant was collected and stored at −80°C. Protein concentrations were determined by Bradford assay using Roti Quant solution (Carl Roth, Karlsruhe, Germany). For SDS-Page, NuPAGE SDS Buffer and NuPAGE Novex Mini Gels (Thermo Fisher Scientific) were used according to the manufacturer's instructions. Western blotting on nitrocellulose was carried out using iBlot dry Blotting System and iBlot Gel Transfer Stacks (Thermo Fisher Scientific) according to the manufacturer's protocol. Blots were probed with antibodies to CD137, CD137L, Vimentin, TLR-7, pAkt, pErk, and β-actin. Antibodies to CD137 and CD137L were obtained from abcam. Vimentin, TLR-7, pAkt, pErk, and β-actin antibodies were purchased at Cell Signaling Technology (Danvers, MA, USA). Anti-mouse IgG and anti-rabbit IgG horseradish peroxidase (HRP) conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Detection and quantification were performed using a Bio-Rad ChemiDoc Touch Imaging System (Bio-rad, Hercules, Ca, USA) with an ImageLab Touch Software Version 1.2.0.12 (Bio-rad). Relative optical density (ROD) was expressed as values for proteins of interest in relation to values of β-actin loading controls.

Luminex analysis

To preserve cell surface epitopes colon cancer cells were carefully detached using accutase solution (Sigma-Aldrich) and 5×10^5 cells were seeded in six-wells plates pre-coated with 10 µg/ml CD137-Fc at 4°C overnight. To determine the protein release of VEGF, PDGF, and sCD137, Luminex analysis was accomplished using Milliplex Map Kits (Merck Millipore, Darmstadt, Germany) according to the manufacturer's instructions.

MTS proliferation assay

To investigate the effect of CD137L activation on tumor cell proliferation of HT-29, HCT-116, SW480, and SW620 cells, 6000 cells/well (HT-29 and HCT-116) or 10,000 cells/well (SW480 and SW629) were seeded in 96 well plates pre-coated with 10 µg/ml CD137-Fc at 4°C overnight. For additional analysis of TLR7 stimulation, cells were treated daily with the TLR7 (and TLR8) agonist R848 (10 µg/ml). 24, 48, and 72 h after seeding cell viability was measured using Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega, Mannheim, Germany) according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed using SPSS Statistics. Overall survival was defined as the time between randomization and death of any cause. Patients who were lost to follow-up were censored at the date of last contact. The overall survival was evaluated by means of PROC PHREG (Cox Proportional Hazards Model). The parameters of prognostic potential, identified in a stepwise procedure, have been further investigated by Kaplan–Meier method (PROC LIFETEST). Frequency distributions were compared using kxm tables (Chi-squared). The log-rank test (with $\alpha = 0.05$) was used to test differences in survival between groups. All statistical analyses were done using the program R (version 3.4.4). Mean cut-off value for low and high expression of CD137 and CD137L in PBMCs were set as fold difference (FD) ≤ 2.0, FD > 2 and FD ≤ 2.9, FD > 2.9, respectively. Mean cut-off values for low and high expression of CD137 and CD137L in tumor tissues were set as FD ≤ 30, FD > 30 and FD ≤ 17, FD > 17, respectively. Pearson's correlation coefficient was used to test and describe bivariate correlations. $p < .05$ was considered statistically significant.

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Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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