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Carcinoembryonic Antigen Cell Adhesion Molecule 1 long isoform modulates malignancy of poorly differentiated colon cancer cells

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Abstract

Objective—Nearly 20%–29% of patients with colorectal cancer (CRC) succumb to liver or lung metastasis and there is a dire need for novel targets to improve the survival of patients with metastasis. The long isoform of the Carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1-L or CC1-L) is a key regulator of immune surveillance in primary CRC, but its role in metastasis remains largely unexplored. We have examined how CC1-L expression impacts on colon cancer liver metastasis.

Design—Murine MC38 transfected with CC1-L were evaluated in vitro for proliferation, migration and invasion, and for in vivo experimental liver metastasis. Using shRNA silencing or pharmacological inhibition, we delineated the role in liver metastasis of Chemokine (C-C motif) Ligand 2 (CCL2) and Signal Transducer and Activator of Transcription 3 (STAT3) downstream of

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Contributors AA was responsible for acquisition of data, analysis and interpretation of data and drafting of the manuscript. JD-C, VB, SH, SY, CT provided technical and material support. KM, CMTG and UDA analysed the TCGA data. MS, RSB and PTG provided cells, reagents and inhibitors, and discussed study design. AA and NB were responsible for the study concept and design, and wrote the manuscript.

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Dedication: This manuscript is dedicated to the memory of Rosalind Goodman.

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CC1-L. We further assessed the clinical relevance of these findings in a cohort of patients with CRC.

Results—MC38-CC1-L-expressing cells exhibited significantly reduced in vivo liver metastasis and displayed decreased CCL2 chemokine secretion and reduced STAT3 activity. Down-modulation of CCL2 expression and pharmacological inhibition of STAT3 activity in MC38 cells led to reduced cell invasion capacity and decreased liver metastasis. The clinical relevance of our findings is illustrated by the fact that high CC1 expression in patients with CRC combined with some inflammation-regulated and STAT3-regulated genes correlate with improved 10-year survival.

Conclusions—CC1-L regulates inflammation and STAT3 signalling and contributes to the maintenance of a less-invasive CRC metastatic phenotype of poorly differentiated carcinomas.

Colorectal cancer (CRC) is a major disease affecting approximately 5% of the population in North America.[1] Approximately 60% of patients survive more than 5 years, but the remaining 20%–29% (USA and Europe, respectively) develop fatal liver or lung metastasis.[2] Novel molecular-based interventions or new surgical techniques for liver resections have been somewhat successful in prolonging life, but new molecular targets need to be identified for better therapeutic management. *C*arcino*E*mbryonic *A*ntigen *C*ell *A*dhesion *M*olecule 1 (CEACAM1, herein abbreviated CC1) is an intercellular adhesion molecule of the immunoglobulin superfamily and a carcinoembryonic antigen (CEA) family member.[3] Many alternate splicing isoforms derived from the human and murine *Ceacam1* gene are tethered to the cell membrane and include either short (S) or long (L) cytoplasmic domains.[3] CC1-L contains two Tyr residues, positioned in Immunoreceptor Tyr Inhibition Motifs (ITIMs),[4] both phosphorylated upon activation of the insulin receptor, the epidermal growth factor receptor, the granulocyte colony-stimulating factor receptor and Src-like kinases.[3] Upon its Tyr phosphorylation and binding to the Src-Homology region 2 domain Tyr phosphatases SHP-1[4] or SHP-2,[5] CC1-L downregulates regulatory signalling pathways,[6] leading to intercellular adhesion regulation,[7] insulin and lipid metabolism,[89] angiogenesis,[10] innate and adaptive immune responses[11–13] and microbial and viral pathogen interactions.[6]

In tumours, CC1-L acts as tumour growth inhibitor in many early solid human neoplasms, including colon tumours.[3] This effect is mediated by the 2 Tyr-phosphorylated residues binding to SHP-1 as shown in colon[41415] and prostate[16] xenografts or allografts. In vivo, $Cc1^{-/-}$ mutant mice demonstrated augmented colon or intestinal tumour burden relative to controls upon azoxymethane treatment[17] and in an $Apc^{1638N/+}$ genetic background.[18] However, in aggressive non-small-cell lung, thyroid, gastric, pancreatic cancers, malignant melanoma and metastatic colon cancer,[3] CC1 overexpression correlates with increased invasiveness, metastatic spread and an unfavourable patient prognosis. In addition to its effects in epithelial cells, CC1-L signalling plays a significant role in invasive processes via endothelial cells,[1019] $CD11b^{+}Gr1^{+}$ immature myeloid cells,[2021] matrix metalloproteinase 9-positive leucocytes and stromal cells.[22]

Murine CRC MC38 cells null for CEACAM1 are highly invasive and metastatic,[21] and thus the present study focuses on poorly differentiated CEACAM1-null colorectal adenocarcinomas. We have now addressed how the CC1-L isoform or a CC1-L mutant,

disabled for Tyr phosphorylation (CC1-FF), expressed in CRC MC38 cells impacts on the development of syngeneic metastatic lesions in vivo. MC38-CC1-L cells demonstrated reduced in vitro proliferation, migration and invasion and reduced metastasis in vivo relative to CC1-negative MC38-Control (CT) cells in immunocompetent wild-type (WT) mice; CC1-L's reduced metastasis phenotype depended on CC1-L's ITIMs, as demonstrated using the MC38-CC1-FF cells. Investigation of the CC1-L-associated regulatory network in MC38 liver metastatic inhibition revealed that CC1-L expression impairs STAT3 activity, thus decreasing the synthesis and secretion of its CCL2 target[23] that controls metastatic immune infiltration.[24] Bioinformatics analysis of The Cancer Genome Atlas (TCGA) tumour samples of CRC patients for CC1 high-expressing tumours demonstrated statistical correlation with some inflammation-regulated and STAT3-regulated genes, with such patients showing improved survival.

MATERIALS AND METHODS

Cell lines and cell culture

Metastatic mouse MC38 colon cancer cells[25] were separated through fluorescent activated cell sorting for CC1-negative expression and retrovirally infected with virions expressing the CC1-L WT and CC1-FF mutant constructs.[15] CCL2-knockdown MC38-CT cells (CCL2-KD) were generated by infecting lentiviral shRNA constructs targeting CCL2 (Open Biosystems; Huntsville, Alabama, USA) under Hygromycin B selection (500 μg/mL). MC38-CTcells were treated with either the S3I-201 STAT3 inhibitor (100 μM in dimethyl sulfoxide (DMSO); Sigma-Aldrich, St-Louis, Missouri, USA), or the SH-0454 and SH-08100 STAT3 SH2 domain-binding inhibitors[26] (1–10 μM in DMSO).

In vivo experiments and metastasis induction

All experimental animal procedures were conducted in compliance with McGill University and the Canadian Council on Animal Care guidelines. Eight-week-old to ten-week-old C57Bl/6 WT mice (Harlan; Frederick, Maryland, USA) and *Ccr2*$^{-/-}$ mice (Jackson Labs; Bar Harbor, Maine, USA) were used for liver metastases development after intrasplenic injection of 2×10^5 viable MC38-derived cells in 50 μL of phosphate-buffered saline (PBS), followed by splenectomy 3 min after injections to prevent sepsis from splenic tumour growth. PBS-injected mice served as controls. Mice were sacrificed 14–21 days post-injection and liver tissue was excised and processed as described in online supplementary materials and methods. C57Bl/6 mice were intraperitoneally (IP)-injected with STAT3 inhibitors 3 days/week for 14 days using DMSO as a control, starting 2 days prior to cell injections.

Histology and immunofluorescence

We determined the number and average size of metastatic lesions, computed with ImageScope software, and reported as area fraction (ratio of surface nodule area/total surface area of liver).[21] For detection on fluorescence or confocal microscopes,[21] frozen sections of the same liver tissues were stained with primary antibodies (see online supplementary materials and methods), followed by incubation with FITC-conjugated or

Texas Red-conjugated antibodies and with 4',6-diamidino-2-phenylindole-containing mounting medium (DAKO; Burlington, Ontario, Canada).[21]

Proliferation, migration and invasion assays

MC38-CT or MC38-CC1-L or MC38-CC1-FF cells were plated in a 16-well E-Plate or CIM-Plate (ACEA Biosciences; San Diego, California, USA) and proliferation, migration and invasion were measured using a real-time xCELLigence instrument (Roche; Basel, Switzerland). Fetal bovine serum (10%) was used as a chemoattractant in migration and invasion assays, with serum-free medium (SFM) as a negative control. Invasion assays were performed using Matrigel-coated CIM-Plates.

Cytokine/chemokine immunoassays

Cell lysate proteins were prepared, the concentrations determined and proteins were subjected to Quansys 9, 16 cytokine/chemokine Multiplex ELISA arrays (Quansys Biosciences; Logan, Utah, USA) (n=3 independent experiments).[21]

Immunoblot analysis

Cell pellets were lysed[21] and equal concentrations of proteins were resolved on SDS-PAGE gels, transferred to polyvinylidene fluoride membranes and immunoblotted with antibodies described in online supplementary materials and methods.

Analysis of patient samples using TCGA

The mRNA sequencing data were downloaded from the BROAD firehose website, using the September 2014 release, and analysed through the RNA-Seq by Expectation-Maximization (RSEM) processing pipeline. The TCGA database does not indicate whether the mRNA was obtained from the invasion front or the luminal side of tumours, which limits comparisons with the work of Ieda *et al.*[27] Analysis of 409 colon and rectal carcinomas sequenced on both Illumina HiSeq and GA platforms was performed to assess the influence of gene signatures on survival. Details on normalisation methods are provided in online supplementary materials and methods. Linear regressions examined associations between clinicopathological variables and quantile-normalised gene expression. Cox proportional hazards analysis was performed to estimate the effect of each gene or signature on survival time and also to estimate whether there was evidence for interaction between high CEACAM1 expression and expression of other signature genes on survival.

Statistical analysis

Data were expressed as mean±SE. Statistical analysis was performed using GraphPad Prism 5 statistical software for Microsoft Windows and R (http://www.cran-r.project.org). The Student two-tailed t and analysis of variance with Bonferroni correction tests were used to determine the significance, and p values <0.05 were considered significant. Survival analysis used Cox proportional hazards models and Kaplan–Meier curves (see online supplementary materials and methods) with significance set at 0.1.

RESULTS

CC1-L expression in MC38 CRC cells decreases invasion in vitro, and liver metastasis in vivo

Depending on the genetic background of CRC cells, CC1 splice isoform expression results in different growth and migratory phenotypes.[1427] We have examined the behaviour of highly metastatic C57Bl/6 MC38 CC1-negative (CT) CRC cells[21] and those expressing the mouse CC1-L isoform or a Tyr-phosphorylation-disabled CC1-L mutant (CC1-FF). MC38 cell-sorted CC1-L populations (see online supplementary figure S1A,B) consistently demonstrated reduced proliferation, migration and invasion (figure 1A–C; quantifications in online supplementary figure 1C–E) relative to CT or to CC1-FF cells. CRC cell chemotaxis was not induced in SFM (figure 1B, C). We performed MC38-mediated liver metastasis assays (intrasplenic injections) in WT B6 mice. MC38-CC1-L cells reduced metastatic burden relative to MC38 CT cells (83% or 96% decrease in number or size, respectively) (figure 1D, E). CC1-L Tyr residues within the ITIM motifs significantly contributed to this decrease as MC38-CC1-FF infectants partially reversed this phenotype with 50% reduction in metastatic burden compared with MC38-CT cells (figure 1D, E). Proliferation (Ki-67), vascular density (CD31) and immune infiltration ($F4/80^+$ macrophages, $CD11b^+$ monocytes and $CD3^+$ and $CD8^+$ T lymphocytes) detected via immunostaining of metastatic nodules indicated that these parameters were significantly diminished in the MC38-CC1-L-derived metastases and partially rescued in MC38-CC1-FF-induced nodules (see online supplementary figure S1F–K). Therefore, CC1-L expression modifies intrinsic MC38 signalling events through its two Tyr residues, leading to inhibition of liver metastatic development.

Relationship of the CCL2-CCR2 signalling axis and CC1-L-mediated decreased metastasis

To examine CC1-L-driven contribution of MC38 tumour cells to immune infiltration, CT, CC1-L- and CC1-FF-transfected cell medium was assessed using ELISA for a panel of 16 cytokines/chemokines (see online supplementary figure S2A). Only CCL2 varied significantly between cell types. MC38-CT cells significantly expressed the CCL2 chemokine mRNA at 72 h post-plating relative to MC38-CC1-L cells (figure 2A). CCL2 was abundantly detected in MC38-CT cell medium, whereas CC1-L-expressing cells exhibited significantly decreased expression; CC1-FF cells had a 2.4-fold enhancement of CCL2 versus that of CC1-L cells (figure 2B), implying that CC1-L acts a negative regulator of CCL2-mediated immune recruitment in the metastatic process.

CCL2 plays a key role in infiltration of $CD11b^+Gr1^{mid}$ myeloid cells into the CRC hepatic metastatic microenvironment,[24] and CCL2 expression in human stage 4 CRC correlates with higher metastatic burden, lower patient survival and recruitment of $CCR2^+Ly6C^+$ monocytes to lung metastases.[28] To explore whether CCL2 was related to CC1-L signalling, we silenced *Ccl2* mRNA in MC38-CT cells with a *Ccl2*-specific shRNA (CCL2-KD) relative to a control (CT-KD) shRNA, which resulted in approximately 60% CCL2 expression relative to those in MC38-CT or -CT-KD cells as defined by ELISA assays (figure 2C). The CCL2-KD cells produced 3.4-fold less metastases in vivo than the CT-KD cells, the former exhibiting similar levels to those of MC38-CC1-L cells (figure 2D). Immune cell profiling of

metastatic livers indicated inhibition of F4/80$^+$ macrophage infiltration, while CD11b$^+$Gr1$^+$ myeloid cell were significantly present in CCL2-KD metastases, suggesting decreased immunosuppression in response to CCL2 silencing (see online supplementary figure S2B,C). This may depend on lack of recruitment of myeloid cells as experimental metastasis performed on a *Rag1*$^{-/-}$ mouse background with CCL2-KD cells still produced a lower metastatic score relative to controls (see online supplementary figure S2D). Alternatively, both natural killer cells[29] and dendritic cells,[30] functional in normal *Rag1*$^{-/-}$ mice, may contribute to reduced metastasis formation in the context of CCL2-KD and CC1-L cells. However, as reported by Wolf *et al*,[28] in later metastasis evaluation, mice injected with CCL2-KD cells did not show significant differences to those of CT-KD MC38 cells (figure 2E). Yet, mice injected with MC38-CC1-L cells still exhibited lower metastatic score than those injected with CT-KD cells (figure 2E), indicating that the CCL2 downregulated activity might be overcome by other targets during late hepatic metastasis such as CCL5, representing another STAT3 target (figure 3C, E), that contributes to in vivo CRC liver metastasis.[31]

The CCL2 receptor, CCR2, is expressed on both CD11b$^+$Gr1$^+$ myeloid cells and endothelium.[2428] To gauge whether the host CCL2-CCR2 signalling pathway might regulate CC1-L-mediated decreased metastasis, we performed metastasis experiments in *Ccr2*$^{-/-}$ and WT mice. MC38-CT and MC38-CC1-L-transfected cells expressed minimal amounts of *Ccr2* mRNA and protein at their cell surface (see online supplementary figure S2E,F). As reported,[28] MC38-CT cells (either non-infected or CT-KD) formed less metastases in the *Ccr2*$^{-/-}$ background than in WT mice (figure 2F). No apparent differences were noted between MC38-CC1-L cells in mice of either genetic background, whereas CCL2 silencing caused lower metastatic development in *Ccr2*-null animals (figure 2F). Hence, our results confirm that MC38-expressed CC1-L decreases CCL2 signalling, which contributes, in part, to lowered metastatic development.[2428]

STAT3 contributes to CC1-L-mediated decreased metastasis

Pan and Shively demonstrated that CC1 deletion in neutrophils altered STAT3 activity and hampered granulopoiesis.[13] Since activated STAT3 is associated with clinical outcomes of patients with adverse CRC,[32] we examined STAT3-mediated signalling changes in MC38-transfected cells. STAT1, STAT6, NFκB, Akt or Erk activities were not different between these cell lines, as measured with specific antiphospho-antibodies (figure 3A). However, pSTAT3 activity followed the same pattern as CCL2 expression, that is, significantly decreased in MC38-CCL1-L cells and returned to baseline in MC38-CC1-FF cells relative to MC38-CT cells (figure 3A, B). These results suggested that a signalling loop might possibly exist between CC1-L as a phosphorylation target of, or coreceptor to, Tyr Kinases or cytokine receptors.[3] Thus, CC1-L could negatively influence STAT3 transcriptional activity,[13] which has been shown to positively regulate CCL2 expression by binding to the *Ccl2* promoter.[23] Treatment with novel STAT3 inhibitors targeting the STAT3 homodimerisation interface leads to breast cancer and glioma[26] cell growth inhibition, apoptosis induction and inhibition of STAT3-regulated genes, including *Ccl2*.[33] We thus treated MC38-CT cells with the S3I-102 STAT3 inhibitor, used mainly for in vitro studies, which considerably reduced both MC38-CT CCL2 and CCL5 chemokine expression at 48

or 72 h post-treatment (figure 3C). At these time points, pSTAT3 activity was completely abrogated in MC38-CT cells, as compared with vehicle-treated MC38-CC1-L cells (figure 3D, top panel). Therefore, reduced CCL2/CCL5 expression in MC38-CC1-L cells is likely caused by the decreased pSTAT3 activity observed.

To confirm that inhibition of STAT3 activity led to reduced CRC liver metastasis in vivo, MC38-CT cells were then treated with two potent and selective, direct-binding STAT3 derivatives of the BP-1-102 compound (SH-0454[26] and SH-08100, chemically modified for better potency) shown to inhibit human breast and lung cancer xenograft growth in vivo.[34] Treatment of MC38-CT cells in vitro with either the SH-0454 or SH-08100 inhibitors (5 μM) led to dramatic reduction of STAT3 activity and CCL2/CCL5 chemokine secretion (figure 3D, bottom panel, and E) as well as decreases in cell migration in vitro (see online supplementary figure S3D), although lower concentrations did not modify MC38-CT cell proliferation or migration over several days (see online supplementary figure S3A, B), due to moderate or no reduction of pSTAT3 levels (figure 3D, bottom panel). These STAT3 inhibitors also exhibited a long-lasting effect in vitro: MC38-CT cells treated for 2 h with the inhibitors (5 μM), which were then completely removed, demonstrated no changes in MC38-CT proliferation but a significant reduction in migration over 30–45 h (see online supplementary figure S3C,D). MC38-CT cells treated for 2 h with either STAT3 inhibitors, then intrasplenically injected into C57Bl/6 mice, showed considerably reduced metastatic load (66% inhibition for SH-0454 and 45% inhibition for SH-08100) compared with mice bearing vehicle-treated MC38-CT cells (figure 3F). Additional thrice weekly injections (IP) of the SH-0454 inhibitor did not result in further decreases in in vivo metastasis (data not shown), most likely due to low blood concentrations of the inhibitor (313±8 nM, 30 min post-injection of 10 mg/kg).[26] Hence, in vivo liver metastasis induced by MC38-CT cells is significantly reduced via decreased STAT3 activity, leading to attenuated CCL2/CCL5 expression.

Survival of patients with higher CC1-L expression

Our data suggested that CC1-L expression in some types of CRC tumours might confer favourable patient outcome through modulation of CCL2 and STAT3. CCL2 expression increases with CRC disease stages and high CCL2 levels significantly correlate with lower disease-free survival.[35] Furthermore, CCL2/CCR2 expression promotes the recruitment of $CD11b^{+}Gr1^{+}$ myeloid cells to favour CRC metastatic development.[24] In addition, STAT3 mRNA and both unphosphorylated and phosphorylated STAT3 protein expression in paired primary and invasive CRCs correlated with lymph node status.[36] Finally, CRC STAT3 activation (pSTAT3) revealed by immunohisto-chemistry was positive in 54% (T3) and 35% (T4) of the large 724 CRC case study evaluated in two prospective cohorts.[32]

To corroborate whether CC1-L cooperated with CCL2/CCR2 and STAT3 in defining overall patient survival, we analysed 409 colon and rectal tumour samples of all stages from the TCGA RNA-Seq data for expression of key genes over a 10-year survival period. As STAT3 activation depends on Tyr750 phosphorylation of the protein and dimerisation resulting in nuclear translocation to activate transcription of target genes,[37] we relied on STAT3 gene

signatures to measure STAT3 activity in patient samples, (see online supplementary table S1).

Analysis of the associations between clinicopathological patient parameters and CEACAM1, CCL2, CCR2 and STAT3 gene signature expression was performed with univariate (see online supplementary table S2, available online only) and multivariate (table 1) linear regression models. The STAT3 expression signature was associated with more advanced age in both univariate and multivariate analyses. Although the presence of venous invasion was associated with CEACAM1, CCL2 and CCR2 when tumours of all stages were analysed in a univariate analysis, only CCR2 expression was associated with venous invasion when a multivariate model was applied, suggesting it as the dominant associated factor for this phenotype. In a subgroup analysis of patients over the age of 60 years, CEACAM1 expression showed association with primary lymphatic invasion (p=0.012 for stages T3–T4; p=0.039 for T1–T4).

We then queried whether the CC1, CCL2 and CCR2 gene expressions were predictive of overall survival; Kaplan–Meier survival analyses for high/low CC1, CCL2 and CCR2 expression did not reveal significant overall 10-year survival differences for patients with T3-T4 CRCs, although those with higher CCR2 expression exhibited a trend towards longer survival (P=0.08). Similarly, when all CRC stages were considered, patients with lower CCL2 expression tended towards longer 10-year survival (p=0.10) (see online supplementary figure S4A–C).

To gauge whether genes relating to STAT3 signatures (see online supplementary table S1) were linked to survival, Cox hazard proportional survival analyses were evaluated with and without an interaction with high/low CC1 expression, including all covariates presented in table 1. Hazard ratios (HR) from the Cox models for each gene (table 2 and see online supplementary table S3) indicate the probability of cancer-associated death. Values imply that higher (>1.0) or lower (<1.0) rates of death, respectively, are associated with higher gene expression of the indicated gene. For interaction models, the interpretation is similar, although the HRs can be viewed as the additional impact of high expression of the indicated gene when CEACAM1 is also high. For the Z-normalised gene expression values, a one-unit change is equivalent to a change of one SD, but for the quantile normalisation the interpretation will vary from gene to gene. Independent STAT3 signature genes included *CCND1, FAS, IL6, IRF1, NPC1* and *STAT1*, suggesting that both inflammation-related and STAT3-related genes statistically influence survival, highlighted by the exponential of the survival coefficient (see online supplementary table S3). Other than IL6, all of these genes exhibited an HR (<1.0) indicative that lower probability of death was associated with higher expression.

When these same gene signatures were analysed with the added interaction of CC1 high/low expression, four genes (*IRF1, MCL1, NPC1* and *SOCS3*) showed statistical significance, emphasising that higher CC1 expression regulates the effect on time to death of genes regulating inflammation (*IRF1*) and STAT3 activity (*MCL1, NPC1* and *SOCS3*) (table 2). However, these associations were not strong. We also performed permutation analyses and the number of small p values did not exceed what might have been expected by chance.

Nevertheless, patient cohorts with advanced CRC tumours exhibiting high CC1 expression significantly correlate with some inflammation-regulated and STAT3-regulated genes and the results are suggestive of longer survival.

DISCUSSION

CC1 is one of the members of the large CEA family of which three proteins (CEA, CEACAM6 and CC1) are known for their contribution to CRC development, progression and metastasis.[3] Although CC1 is downregulated in benign CRC tumours,[3] Ieda *et al*[27] have shown that CC1 re-expression and, in particular, CC1-L isoform dominance over CC1-S at the invasion front correlates with CRC haematogenous metastasis. These data suggest that different CC1 isoforms expressed in 75 CRC patients in stages 3 and 4 over a 5-year survival period affect CRC metastasis. However, the CC1-L-governed signalling and mechanisms during in vivo metastasis have remained so far unexplored. We have now examined, using the murine MC38 CEACAM1-null cell line producing poorly differentiated adenocarcinoma, how CC1-L regulates a signalling network, operative in the context of a syngeneic mouse background.

CC1-L expression in MC38 cells resulted in a considerable decrease of CRC liver metastatic burden. In search of mechanisms responsible for the observed phenotype, we first identified that the CCL2 chemokine expression was decreased in MC38-CC1-L cells. CCL2 silencing led to significantly reduced metastatic development at early (14 d) but not late (20 d) time points. Similarly, ablation of the CCL2-CCR2 axis in *Ccr2*$^{-/-}$ mice only partially protected mice against liver metastasis, as described.[2428] It remains possible that CCL2 may also bind to the atypical chemokine receptors ACKR1 and R2 mediating CCL2 signalling in leucocytes,[38] but this alternative pathway is yet to be investigated in liver metastasis. Importantly, CC1-L-mediated stromal effects are also implicated in the CRC metastatic phenotype with dampened CCL2 secretion leading to decreased CD11b^{+} and F4/80^{+} myeloid cell infiltration to the metastatic site.[21] Inflammatory chemokines such as CCL2 and CCL5 play a significant role in driving CRC metastatic processes.[39] In fact, discontinuation of an anti-CCL2 treatment in breast cancer models leads to accelerated metastasis through angiogenesis stimulation.[40] Although high or low CC1 expression is not predictive of 10-year overall survival in patients with stages 3 and 4 CRC, as predicted by Kaplan–Meier survival curves, patients with low CCL2 or high CCR2 expression have a marginally better survival.

STAT3 is also associated with inflammatory CRC processes[37] and CC1-L, possibly through the latter's role as a coinhibitory receptor to Tyr kinase,[38] or to cytokine receptors regulating canonical STAT3 signaling[13] acts on STAT3 inflammation-related signalling pathways. It remains possible that CC1-L might also signal through other unexplored non-canonical signalling pathways.[37] The IL-6 receptor, as a major CRC STAT3 signalling cytokine receptor[37] is not expressed in MC38 cells (data not shown), rendering its triggering by IL-6 non-operative. But it remains possible that IL-11 secretion by the MC38-CC1-L cells might activate the GP130/STAT3 signalling and reduce metastasis;[41] this remains to be further evaluated.

Pharmacological inhibition of STAT3 activity in MC38-CT cells resulted in the abrogation of CCL2 and CCL5 chemokine secretion reduced in vivo liver metastatic development. These findings recapitulated the diminishment in STAT3 activity and CCL2/CCL5 chemokine levels observed in MC38-CC1-L cells (figure 3). Importantly, a 2 h exposure of MC38 cells to new STAT3 inhibitors demonstrated stable dampening of MC38 STAT3 activity in vivo. The low bio-availability of these compounds[42] is currently being improved through chemical modifications. As STAT3 promotes resistance to widely used Tyr kinase inhibitors in lung cancer and CRC, and since its silencing reinstated drug sensitivity,[43] these STAT3 inhibitors may constitute novel therapeutic compounds that are able to reverse drug resistance.

Previous findings have indicated that activated STAT3 represents a biomarker for poor survival outcome.[32] In this analysis of TCGA CRC samples, we demonstrate that high CC1 expression combined with four of the STAT3 signature genes expressed during inflammation were predictive of longer survival times (table 2). Importantly, one of these genes is *SOCS3*, a known STAT3 inhibitor.[37] The power of survival analysis rests on the number of deaths, and therefore power was limited in the TCGA data. Investigation of these relationships in a larger dataset would be worthwhile.

However, our data do not necessarily imply a direct regulation of these activities by CC1-L; this modulation could be executed through tiers of other events/molecules that interconnect CC1-L to other metastasis-promoting pathways. Furthermore, human CRC cell lines and tumour tissues often abundantly express two other CEACAM proteins, CEA and CEACAM6;[3] these genes share 70%–79% of the proximal promoter sequences with that of *CEACAM1*. The hierarchy of CEACAM functions and predominance under static conditions has been partially addressed[44] and this will need to be further pursued in plastic and modulated culture conditions in CRC cell lines with defined genetic backgrounds; this constitutes our challenge of the next few years.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Significance of this study

What is already known on this subject?

▶ Liver metastasis of colorectal cancer (CRC) is a major cause of death in patients with cancer.

▶ Carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) is downregulated in early colorectal tumours and CEACAM1-L is present at the invasion front of CRC cells. However, the mechanism through which CEACAM1 impacts metastatic dissemination of CRC cells to the liver has not been investigated to date.

▶ CCL2–CCR2 interactions and STAT3 activity influence breast and colon cancer metastases.

What are the new findings?

▶ CEACAM1-L expressed in some metastatic murine CRC cells significantly reduced experimental liver metastatic burden.

▶ CEACAM1-L expression in MC38 cells decreases STAT3 activity and CCL2 secretion, thus regulating inflammatory signalling networks and decreasing metastatic burden.

▶ Patients with stages 1–4 CRC expressing low levels of CCL2 or high CCR2 exhibit a better 10-year survival.

How might it impact on clinical practice in the foreseeable future?

▶ CEACAM1 plays an important role in colorectal carcinoma liver metastasis and may constitute a novel target for therapeutic intervention.

▶ Generation of new Stat3 inhibitors with improved potency and pharmacokinetic properties provide a promising prospect in prevention of cancer metastasis.

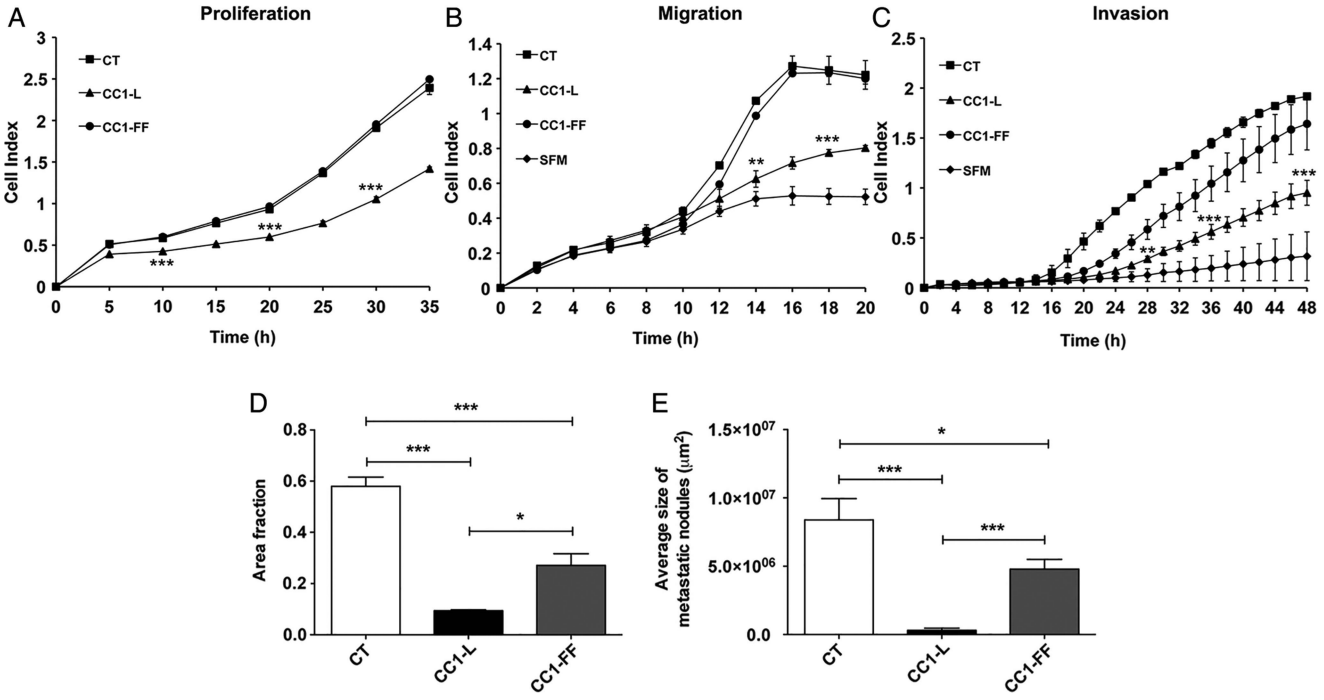

Figure 1.

Carcinoembryonic antigen-related cell adhesion molecule 1 (CC1) expression regulates metastatic capabilities of mouse MC38 colorectal cancer (CRC) cells. (A) Proliferation of MC38-CT, MC38-CC1-L or MC38-CC1-FF cells measured over 35 h using the xCELLigence system. (B) Migration of the same cells was measured over 20 h; serum-free medium (SFM) served as negative chemotaxis control. (C) Cell invasion in Matrigel-coated plates was measured over a 48 h time period with SFM as negative chemotaxis control. (D and E) Metastatic ability of the MC38-derived cells in vivo was examined 14 days after intrasplenic injection into C57Bl/6 mice. The number (D) and size (E) of metastatic nodules in the livers were quantified on H&E-stained step sections of livers. (ns $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$, two-tailed t test or analysis of variance with Bonferroni correction test). Data are presented as means±SEM with n=at least two independent sets of experiments. For in vivo studies, a minimum of 10 mice per group were used.

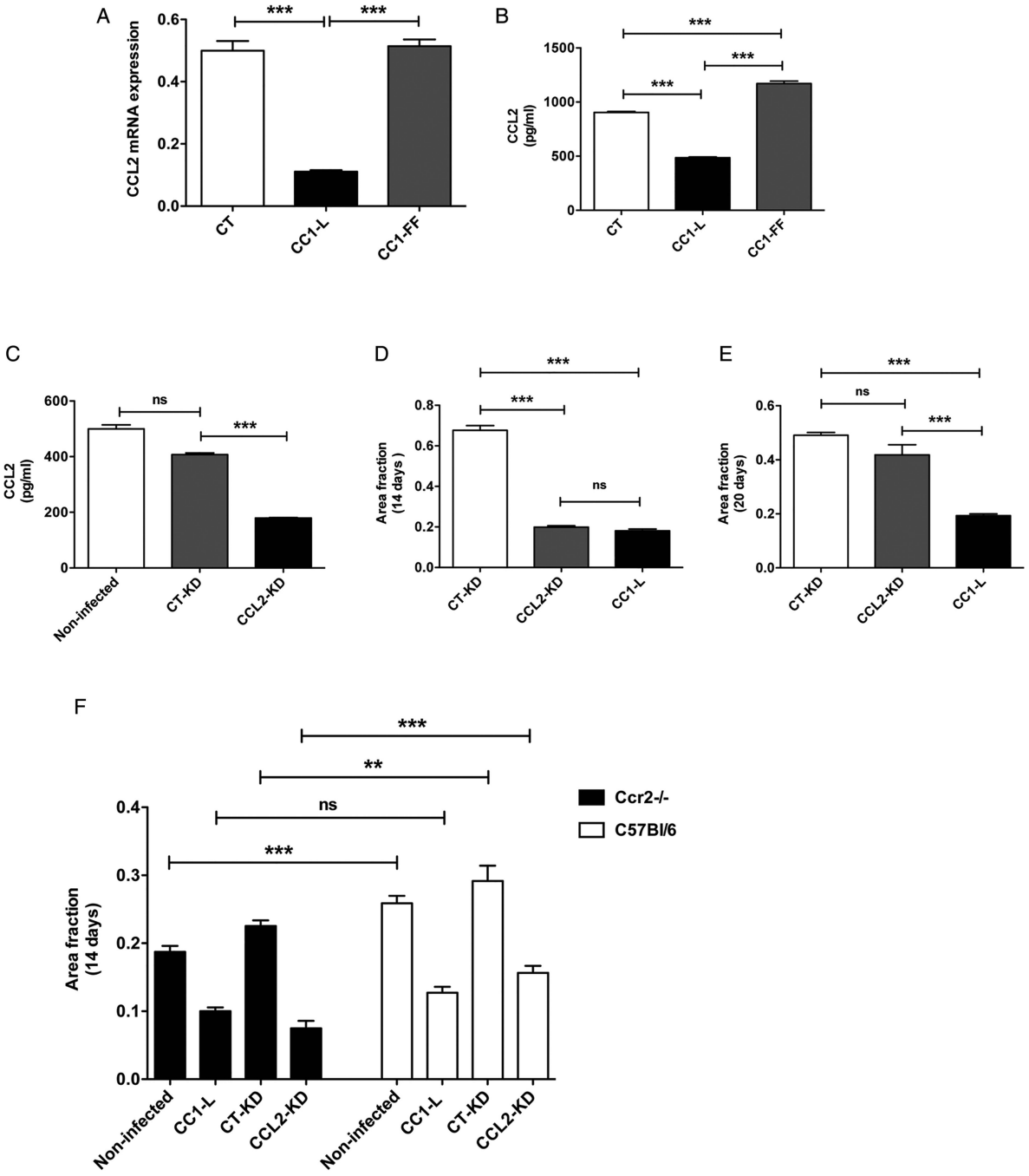

Figure 2.

CCL2-CCR2 signalling axis contributes to anti-metastatic function of CC1-L. (A) MC38-CT, MC38-CC1-L and MC38-CC1-FF cells were grown for 72 h, and CCL2 chemokine mRNA levels were measured using Q-PCR. (B) CCL2 protein levels in cell lysates were measured using ELISA 72 h post cell seeding. (C) MC38-CT cells were stably infected with virions expressing either CCL2 shRNA (CCL2-KD) or control shRNA (CT-KD). CCL2 protein levels in cell lysates were measured using ELISA in non-infected, CT-KD and CCL2-KD cells. (D and E) metastatic ability of MC38-CCL2-KD cells was evaluated in

vivo in comparison with MC38-CT-KD and MC38-CC1-L cells, 14 (D) and 20 (E) days after intrasplenic injections into C57Bl/6 mice. (F) Liver metastasis formation was measured at day 14 in *Ccr2*$^{-/-}$ vs C57Bl/6 backgrounds. MC38-CCL2-KD, MC38-CT-KD, MC38 non-infected and MC38-CC1-L cells were used for experimental metastasis (ns $p>0.05$, *$p<0.05$, **$p<0.01$, *** $p<0.001$, two-tailed t test or analysis of variance with Bonferroni corrections). Data are presented as means±SEM and with n=at least two independent sets of experiments. For in vivo studies, a minimum of 10 mice per group were used.

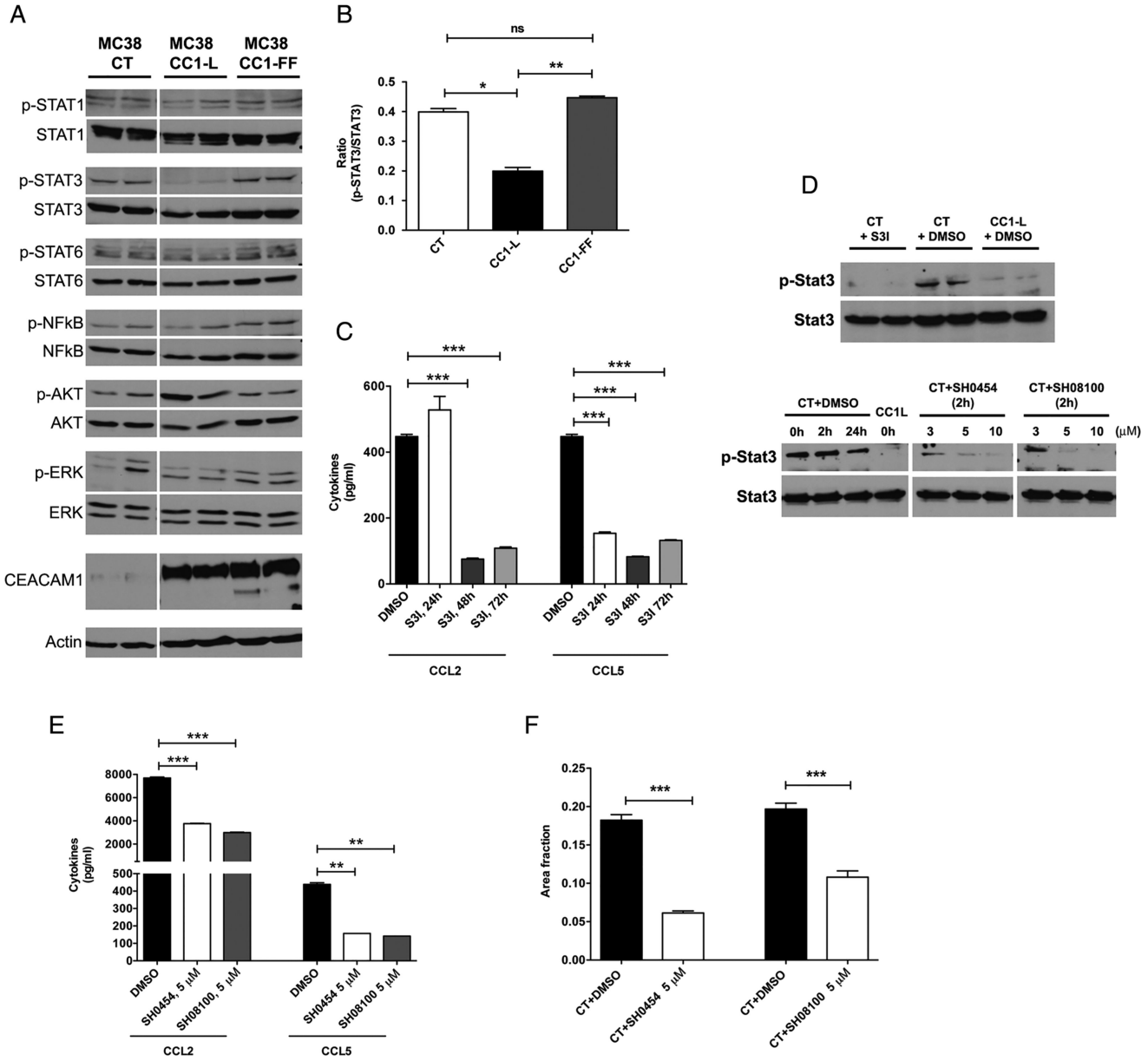

Figure 3.

STAT3 activity regulates liver metastasis downstream of CC1-L signalling and can be targeted with synthetic compounds. (A) Immunoblot analysis of several important signalling proteins in MC38-CT, MC38-CC1-L or MC38-CC1-FF cells. Actin was used as a loading control. (B) Quantification of STAT3 activity is shown as the ratio of p-Stat3 to Stat3. (C and D) MC38-CT cells (showing high level of STAT3 activity) were treated for 24, 48 or 72 h with either commercially available STAT3 inhibitor (S3I-201; 100 μM) or dimethyl sulfoxide (DMSO). (C) CCL2 and CCL5 chemokine levels in cell lysates were measured using ELISA after incubation with the inhibitor and (D, top panel) STAT3 activity was evaluated using immunoblotting. (E) Two new synthetic compounds specifically targeting STAT3 (SH-0454 and SH-08100) were examined for their dose-response inhibitory activity. Different concentrations (3, 5 and 10 μM) of each compound were applied to MC38-CT

cells for 2 h; (D, bottom panel) STAT3 activity was measured by immunoblotting. STAT3 activity in CC1-L cells is shown for comparison purposes. (E) CCL2 and CCL5 levels were also measured using ELISA at median inhibitory concentration (5 μM) of each compound with DMSO used as vehicle control. (F) MC38-CT cells treated with either DMSO or 5 μM of each compound for 2 h were intrasplenically injected into C57Bl/6 mice and liver metastasis was evaluated 14 days post-injections. (ns $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$, two-tailed t test or analysis of variance with Bonferroni correction). Data are presented as means±SEM with n=at least two independent sets of experiments. For in vivo studies, a minimum of 10 mice per group were used.

Table 1

p Values for multivariate analyses of associations between the listed clinicopathological variables and quantile-normalised expression of each of CEACAM1, CCL2, CCR2 and the STAT3 signature genes *

	CEACAM1		CCL2		CCR2		STAT3 signature	
	T3–T4	T1–T4	T3–T4	T1–T4	T3–T4	T1–T4	T3–T4	T1–T4
Age	0.17	*0.039*	0.788	0.411	0.686	0.285	*0.035*	*0.017*
Gender (male vs. female)	0.58	0.37	0.35	0.38	0.16	0.18	0.80	0.70
Primary tumour site (colon vs rectum)	0.23	0.23	0.67	0.65	0.88	0.87	0.34	0.35
Primary lymphatic presentation	*0.092*	0.11	0.68	0.51	0.53	0.34	0.42	0.52
Lymphatic invasion	0.86	0.75	0.45	0.70	0.87	0.61	0.54	0.83
Venous invasion	0.36	0.12	0.69	0.90	0.19	*0.039*	0.80	0.99

* p Values are indicated for association in a multivariate linear model predicting gene expression as a function of the variables listed in the rows of the table. Separate models are fit for each column of the table; statistically suggestive values (<0.1) are indicated in italics.

Table 2

Selected p values and HRs for tests of interaction between genes in any of the signatures and CEACAM1-high expression, from survival analysis models*

Categories†	Gene	T3–T4 stage Quantile	HR coefficient	T1–T4 stage Quantile	HR coefficient
2	*IRF1*	0.042	0.44	0.122	0.57
3	*MCL1*	0.057	0.36	0.005	0.23
3	*NPC1*	0.013	0.17	0.027	0.24
2	*SOCS3*	0.048	0.55	0.086	0.63

*p Values for interaction are indicated and p<0.1 are highlighted.

†Refers to gene categories in Supporting table 1. Gene expression was quantile-normalised, and all covariates in table 1 were included in the models. The HRs corresponding to each p value represent the estimated magnitude of the interactions between each gene and CEACAM1-high. Values <1 indicate lower probability of death and longer survival. Genes were selected for inclusion in this table if there was some (p<0.1) evidence for association with survival.