Bone marrow-derived CD11b+Jagged-2+ cells promote epithelial to mesenchymal transition and metastization in colorectal cancer

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Abstract
Timely detection of colorectal cancer metastases may permit improvements in their clinical management. Here we investigated a putative role for BM-derived cells in the induction of epithelial-to-mesenchymal transition (EMT) as a marker for onset of metastasis. In ectopic and orthotopic mouse models of colorectal cancer (CRC), BM-derived CD11b(Itgam)+Jagged2+ cells infiltrated primary tumors and surrounded tumor cells which exhibited diminished expression of E-cadherin and increased expression of vimentin, two hallmarks of EMT. In vitro co-culture experiments demonstrated that the BM-derived CD11b+Jag2+ cells induced EMT through a Notch dependent pathway. Using neutralizing antibodies, we imposed a blockade on CD11b+ cells recruitment to tumors which decreased the tumor-infiltrating CD11b+Jag2+ cell population of interest, decreasing tumor growth, restoring E-cadherin expression and delaying EMT. In support of these results, we found that peripheral blood levels of CD11b+Jag2+ cells in mouse models of CRC and in a cohort of untreated CRC patients were indicative of metastatic disease. In CRC patients, the presence of circulating CD11b+Jag2+ cells was accompanied by loss of E-cadherin in the corresponding patient tumors. Taken together, our results show that BM-derived CD11b+Jag2+ cells which infiltrate primary colorectal tumors are sufficient to induce EMT in tumor cells, thereby triggering onset of metastasis. Further, they argue that quantifying circulating CD11b+Jag2+ cells in patients may offer an indicator of CRC progression to metastatic levels of the disease.
Introduction
Metastatic disease is a major cause of cancer-associated mortality. Despite significant advances in the treatment of primary tumors, metastases remain a significant clinical problem, likely reflecting our limited knowledge of the mechanisms governing this complex process(1). It is accepted that metastization follows a series of interrelated steps, each of which can be rate-limiting. These steps include: local invasion by tumor cells; entry into systemic circulation (‘intravasation’); invasion of the target organ (‘extravasation’); and finally proliferation and growth of the secondary tumor(2). One of the major processes regulating local invasion in epithelial tumors is termed epithelial to mesenchymal transition (EMT)(3, 4). EMT is a transcriptional regulated transdifferentiation process characterized, at the tumor cell level, by a decrease in epithelial markers such as E-cadherin, loss of cell-cell adhesion, apical-basal polarity and acquisition of mesenchymal markers such as vimentin associated with an increase in cell motility and invasion capacity(5-8). EMT has been positively correlated with increase breast and colon cancer metastasis and decreased patient survival(3)(9, 10).

In the last decade there has been increasing evidence suggesting that tumor metastasis is also regulated by non-malignant cells of the tumor microenvironment, namely by bone marrow (BM) - derived cell populations(11). In fact distinct BM-derived populations such as tumor associated macrophages (TAMs)(12, 13), pre-metastatic niche cells(14) and endothelial progenitor cells (EPCs)(15) have been shown to enhance metastatization via multiple processes. Nevertheless a direct role of BM-derived cells in promoting EMT at the primary tumor has not been described, and was the focus of the present study.

Using ectopic and orthotopic colorectal cancer (CRC) models in mice, we show that a population of BM-derived myeloid (CD11b+F4/80+) expressing Jagged 2 (Jag2) is
actively recruited into colon tumors and accumulates in tumor areas undergoing EMT. Detailed analysis of this tumor:BM-derived cell interaction shows the latter induce EMT via Notch activation on the tumor cells. Importantly, in vivo depletion of CD11b+ cells in ectopic CRC models reduced the recruitment of CD11b+Jag2+ cells into the tumors and significantly decreased EMT. Quantification of circulating (peripheral blood) and tumor-derived CD11b+jag2+ cells in CRC patients was significantly correlated with the presence of metastatic disease. Together, the data presented here reveals a novel undisclosed role for BM-derived cells in inducing EMT in primary CRC and identifies a BM-derived cell population that may be targetable and studied as a biomarker for CRC metastases formation.

Materials and Methods

**Human peripheral blood (PB) samples collection and processing**

PB samples of sequential colorectal cancer patients evaluated at diagnosis by the Multidisciplinary Colorectal Cancer Team were collected at the Gastroenterology Department at Instituto Português de Oncologia, Lisbon, Portugal (IPO) after informed consent and institutional review board approval (IPO, Lisbon, Portugal), in accordance with the Declaration of Helsinki. Patients were included if they had a pathology exam showing colorectal adenocarcinoma. All patients previously submitted to endoscopic, surgical or medical treatment for colorectal cancer were excluded. PB samples were collected in 4 EDTA coated tubes to a total volume of 12 ml. Samples were centrifuged at 4ºC, for 8 minutes at 1500 rpm. Plasma was collected and stored at -80ºC. The remaining fraction was lysed using 50ml of red cell lysis buffer (RCLB) for 20 minutes at room temperature. The resulting
mononuclear cell fraction was washed in PBS EDTA 2mM + 0.5% BSA and used for further analysis. Colorectal cancer patients staging was done according to the American Joint Committee on Cancer (AJCC) Staging System.

**Mouse strains, BM transplants, ectopic and orthotopic colon carcinoma model**

Animal experiments were performed with the approval of the Instituto Gulbenkian de Ciência Animal Care Committee and Review Board. *In vivo* experiments were performed on 4-8 weeks old female Nude mice (C57/BL6 background). For BM transplants Nude mice received a whole body lethal irradiation (800-950 rads) and 24 hours later received an intravenous injection of 2-3x10^6 BM mononuclear cells collected from Actin-GFP male mice (C57/BL6 background). Mice were allowed to recover for 2-4 weeks. After this period peripheral blood samples were collected from the facial vein in EDTA-coated tubes (Multivette 600, Sarstedt, Nümbrecht, Germany) and analysed by flow cytometry for GFP+ cells. Mice were considered suitable for further experiments when the percentage of GFP+ cells in the peripheral blood was over 80% of total cells. Xenografted ectopic colon carcinoma tumors were induced by inoculation of 5x10^6 HCT15, HCT116, DLD-1, or HT-29 cells (human colorectal carcinoma cell lines; these were obtained from ATCC, DC, USA, in 2012 and were not passaged for over 6 months in our Laboratory) subcutaneously in Nude mice. Tumors were allowed to grow and at specific time points (1 to 3 weeks) mice were sacrificed and tumors collected. Tumors were fixed (10% formalin or paraformaldehyde) and included (paraffin or gelatine respectively), frozen at -80°C for further RNA isolation or digested for FACs analysis. Xenografted orthotopic colorectal carcinoma tumors were induced by inoculation of 1x10^6 HCT15 or HCT116 cells, into the visceral cecal wall of Nude mice. PB samples were collected.
from the facial vein at different time points and further processed for flow cytometry analysis. CD11b neutralizing antibody in vivo administration was performed as follows: briefly, 500 μg anti-CD11b (clone 5C6) neutralizing monoclonal antibodies against CD11b were administered intra-peritoneal every 3 days into tumor bearing mice, starting on day 5 post inoculation.

**Isolation of BM-derived cells from the tumors**

Tumor samples were mechanically fragmented into 2mm per 2mm pieces and then digested with collagenase (Sigma-Aldrich, 2mg/ml in serum free DMEM medium) for 1 hour at 37°C and 5% CO₂. After digestion, tumor cell suspensions were passed through a mesh and washed in sterile PBS. Further isolation of tumor cell population was performed by cell sorting using FACSaria (BD Biosciences). Isolation of tumor derived GFP+ population was done without the use of any antibody staining, while isolation of Jagged-2+ and CD11b+ population required previous staining with fluorochrome conjugated antibodies (PE anti-mouse Jagged-2, 131007, BioLegend and FITC anti-mouse CD11b, 101205 BioLegend). Antibodies used were diluted 1:100 in PBS+BSA 0,5% and incubated in the dark with rotation, at 4°C for 45 minutes.

**In vitro co-culture assays**

HCT15 cells were cultured at 1x10⁴/cm² cell density in DMEM supplemented medium (GIBCO) with 10% FBS (foetal bovine serum, Sigma-Aldrich). After 24h medium was changed to DMEM supplemented medium with 2% FBS and 1x10⁵ tumor associated BM-derived cells were added (GFP+, GFP+ Jagged-2+, GFP+ Jagged-2 negative or Jagged-2+ CD11b+ cells). Co-culture was maintained for 48h.
After this period tumor associated BM-derived cells were gently washed from the culture and HCT15 cells collected for mRNA extraction or fixed with 2% paraformaldehyde for 15 minutes for further immunocytochemistry staining. γ-secretase inhibitor or GSI (DAPT, Sigma Aldrich) was added to co-cultures at a final concentration of 10uM and respective controls received DMSO (Sigma-Aldrich).

**Flow cytometry**

PB mononuclear cell fractions derived from CRC patients were stained for CD11b and Jagged-2. Briefly, 5x10⁵ cells were blocked for 10 minutes at 4°C with FcR fragment in a 1:100 dilution and then incubated with anti-CD11b (APC anti-human CD11b, 301409 BioLegend) and Jagged-2 (PE anti-human Jagged-2, 346903 BioLegend) antibodies in a 2,5:100 dilution in PBS+BSA 0,5% for 45 minutes in the dark with rotation at 4°C. Characterization of tumor associated BM-derived cells was performed by staining digested tumor samples for Jagged-2 (PE anti-mouse Jagged-2, 131007, BioLegend), CD11b (FITC anti-mouse CD11b, 101205 BioLegend), Gr-1 (APC/Cy7 anti-mouse Ly-6G/Ly-6C (Gr-1), 108423 BioLegend), F4/80 (FITC anti-mouse F4/80, 123107 BioLegend), Sca-1 (FITC anti-Mouse Ly-6A/E, 557405 BD Pharmingen), c-Kit (APC/Cy7 anti-mouse CD117 (c-kit), 105825, BioLegend), CD3 (FITC anti-mouse CD3, 100203 BioLegend) and CD19 (Alexa Fluor® 488 anti-mouse CD19, 115524 BioLegend). Antibodies used were diluted 1:100 in PBS+BSA 0,5% and incubated in the dark with rotation, at 4°C for 45 minutes. Flow cytometry was performed on FACSCalibur and analyzed with FlowJo 8.7 Software (Tree Star, Inc. 1997 - 2012).

**Quantitative Reverse-transcription Polymerase Chain Reaction (qRT-PCR)**
RNA extraction (Trizol, Invitrogen), cDNA synthesis (Reverse-transcription with Superscript II reverse transcriptase (Invitrogen)) were performed following standard protocols. RT-PCR was performed with Power SYBR Green PCR Master Mix in 7900HT Fast Real-Time PCR System (both from Applied Biosystems, Foster City, CA). Primer sequences for hE-Cadherin, hVimentin, hHey1, hHey2, mDll1, mDll4, mJag1 and mJag2 are shown in Table 2. The housekeeper gene used to normalize human samples was h18s and to normalize mouse samples was m18s. RT-PCR data were analyzed by DataAssist software (Applied Biosystems Foster City, CA).

**Tumor histocytochemistry procedure and analysis**

Human CRC tumor paraffin included samples were provided by the Pathology department at IPO-Lisboa. Samples were serially sectioned (3 μm), adsorbed into slides and then subjected to antigen retrieval (PT Link, Dako). After being deparaffinised, sections were blocked in PBS + 10% Goat Serum for 30 minutes at room temperature and then incubated with primary antibody at room temperature for 1h. Counterstaining was performed using Mayer’s hematoxylin. Serial sections were stained for Jagged-2 (sc-56041 Santa Cruz, 1:15), CD11b (HPA002274 Sigma, 1:300), E-Cadherin (18-0223 Invitrogen, 1:50) and Cytokeratin 19 (M0888 Dako, 1:50). Slides were analyzed and photographed in a Leica DMD108 microscope. Concerning CD11b+ Jagged-2+ cell quantification tissue sections were screened at low power field (x100 magnification), and the 10 areas with the most intense staining for CD11b (hot spots) were selected. Jagged-2 staining in these areas was confirmed in the serial slide. Counts of the hot spots were performed at high power field (x400). The mean number of positive cells in the 10 hot spots areas was expressed for each condition. E-Cadherin quantification on each slide was
performed using the ImmunoMembrane software version 1.0i (16). Results were further validated by a pathologist at IPO – Lisboa. Mouse tumors samples were included in either gelatine or paraffin. Paraffin tumor sections were further subjected to antigen retrieval protocols (PT high). Tumour cryosections were blocked with a 5% FBS / 0.1% BSA solution in PBS for 30 minutes. Slides were then covered with primary antibodies: anti-mouse PECAM (553369, Pharmingen), anti-mouse CD11b (550282, Pharmingen), anti-human E-cadherin (M3612, Dako); anti-human Cytokeratin-19 (M0888, Dako), anti-human (M0851, Dako), anti-human C-Kit (A4502, Dako), anti-mouse B220 (553085, Pharmingen) and anti-human Jagged-2 (AbCam). After overnight incubation at 4ºC, tumor sections were washed in PBS and incubated with secondary antibodies from Invitrogen (anti-rat-FITC, anti-mouse-Alexa568, anti-rabbit-Alexa488, respectively) for 2 hours at room temperature. For the quantification of GFP+ lineage+ cells in tumor samples the total number of GFP+ lineage+ cells was quantified in 5 high power fields (x400) and then divided by total GFP+ cells. vimentin+ cells quantification was performed by direct count of Vimentin expressing cells in 5 high power fields (x400). Quantification of E-cadherin expression on tumor samples was performed by direct quantification of staining intensity and area using ImageJ software.

Statistical Analysis

Results are expressed as mean ± standard error mean. Data were analyzed with GraphPad software (GraphPad Software, Inc; v4.0b) using unpaired two-tailed student's t test or Mann-Whitney test when indicated. P values of <0.05 were considered statistically significant.
Results

**BM-derived cells infiltrate ectopic colorectal tumors and localize in regions undergoing EMT**

To characterize the contribution of bone marrow (BM)-derived cells during colorectal cancer (CRC) progression, we started by inoculating HCT15 (human colon carcinoma cell line) sub-cutaneously into nude mice that had been transplanted with actin-GFP+ BM (Supplementary Figure 1). Tumors were allowed to grow for 1 or 2-3 weeks (termed early and late tumors respectively), after which mice were sacrificed and tumors collected. As shown in Fig 1, BM-derived cells (GFP+ cells) actively infiltrate early and late tumors, and are found at higher frequency in late tumors (Figure 1A). We also observed that as tumors grew there was a significant decrease in E-cadherin expression and a significant increase in the expression of the mesenchymal marker vimentin (Figure 1B and C), suggestive of EMT. Considering that BM-derived cells infiltration into tumors and EMT were associated with tumor growth we analysed possible correlations between GFP+ cell infiltration and E-Cadherin and vimentin expression in tumor tissues. There was an inverse correlation ($P = 0.0097$; $r^2 = 0.7678$, 7 samples) between GFP+ and E-cadherin+ areas, but a direct correlation ($P = 0.0297$; $r^2 = 0.6448$, 7 samples) between GFP+ areas and the number of vimentin+ cells in tumor tissues (Figure 1D). Detailed observation of tumor sections showed that cytokeratin-19 (CK19) positive tumor cells in close proximity with GFP+ cells acquire the expression of vimentin, while tumor cells further away do not (Figure 1E lower panels, white arrows). Consistent with the EMT phenotype, tumor cells in proximity of GFP+ cells also show decreased E-cadherin expression (Figure 1E lower right panel, white arrow). These data suggest that CRC growth is associated with an increased frequency of BM-derived cells infiltration, which in turn correlate with the onset of
EMT. Considering this we hypothesized BM-derived cells could induce EMT on epithelial tumor cells.

**BM-derived cells induce EMT on colorectal carcinoma cells in a Notch pathway-dependent manner.**

To test the ability of BM-derived cells to induce EMT on tumor cells we performed *in vitro* co-culture experiments with HCT15 cells and BM-derived (GFP+) cells isolated (sorted out) from late subcutaneous tumors. As depicted in Figure 2A in the presence of BM-derived (GFP+) cells isolated from tumors (TGFP+ cells), HCT15 lose E-cadherin expression and gain vimentin expression, as measured by qRT-PCR. This can also be observed in the quantification of E-cadherin expressing HCT15 cells by inmunostaining after co-culture (Figure 2B), suggesting that TGFP+ cells induce vimentin expression and decrease E-cadherin expression at both transcriptional and translational level. Next we looked into the molecular pathways regulating EMT induced by TGFP+ cells. The TGF-β and the Notch signalling pathways have been implicated in EMT, thus we tested if these pathways could be involved in BM-derived cell induced EMT on colon carcinoma cells. In agreement we performed co-culture assays adding a TGF-β blocking antibody and a Notch pathway inhibitor (gamma secretase inhibitor, GSI) and determined its effect on HCT15 cells E-cadherin and vimentin expression. TGF-β inhibition had no effect (data not shown), while addition of GSI inhibited the TGFP+ cells-induced decrease in E-cadherin and increase in vimentin expression (Figure 2A). Further confirming the involvement of the Notch-pathway is the observation that HCT15 cells express Notch receptors 1 and 4 (data not shown) and more importantly the Notch pathway downstream targets Hey 1 and 2 are upregulated in HCT15 cells undergoing EMT, in the presence of TGFP+ cells (Figure 2A). These data suggests that BM-derived cells induce EMT via Notch pathway activation on tumor cells.

**BM-derived cells expressing Jagged-2 induce EMT on colorectal cancer cells**

Having shown TGFP+ cells induce EMT on HCT15 cells and that this process involved Notch pathway activation, next we determined which ligands of the Delta:Notch family were expressed by the tumor-infiltrating BM-derived cells. We determined and compared the expression of the ligands Delta-like ligand 1 (Dll1), 4
(Dll4) and Jagged-1 and 2 (Jag1, Jag2) in BM-derived cells (GFP+) collected from late tumors and from the corresponding BM samples. Jagged-2 was the most abundantly expressed ligand on TGFP+ cells, its expression being significantly higher on TGFP+ cells than on those isolated from BM (Figure 3A). Detailed flow cytometry analysis of tumor and BM samples of tumor bearing mice revealed a population of GFP+ Jagged-2+ cells in both tissues, although at a higher frequency in the tumors, representing an average of 5.5 ± 1.12% cells (Figure 3B). Taking into account the high frequency of GFP+ Jagged+ cells in tumors, next we investigated whether this population was able to induce EMT on HCT15 colon carcinoma cells.

GFP+ Jagged-2+ and also GFP+ Jagged-2 negative cells where sorted from late tumors (Figure 3B) and co-cultured with HCT15 in the presence or absence of GSI. As depicted in Figure 3C and D, GFP+ Jagged-2+ but not GFP+ Jagged-2 negative cells were able to reduce E-cadherin transcription in a Notch pathway dependent manner. In accordance, the number of HCT15 E-cadherin+ cells is significantly reduced when these are co-cultured in vitro with tumor GFP+Jagged-2+ (Figure 3C). This phenotype is reversed upon the addition of GSI or when HCT15 cells are co-cultured with GFP+ Jagged-2 negative cells (Fig 3E). Taking together these results suggest that BM-derived cells modulate EMT via Jagged-2 mediated Notch pathway activation on tumor cells.

**BM-derived CD11b+Jagged-2+ cells induce EMT via Jagged-2 mediated Notch pathway activation**

Having shown that BM-derived Jagged-2+ cells are responsible for tumor cell EMT via Notch pathway activation, next we sought to determine the identity of these cells more precisely. The majority of TGFP+ Jagged-2+ cells were CD11b+ (85 ± 1.0%), F4/80+ (64.1 ± 0.55%) and Sca-1+ (52.5 ± 1.55%) (Figure 4A and B). Considering the high frequency of CD11b+ cells within the TGFP+ Jagged-2+ population we tested if tumor-derived CD11b+ Jagged-2+ cells (CD11b+Jag2+) could induce HCT15 EMT in vitro. Accordingly, co-culture of HCT15 tumor cells with tumor derived CD11b+Jag2+ cells led to a significant decrease in E-cadherin expression at both the mRNA and protein level (Figure 4D and E), in a Notch pathway-dependent manner since GSI treatment reverted this effect. Altogether these data suggest that CD11b+Jag2+ BM-derived cells actively infiltrate CRC tumors and induce EMT on tumor cells in a Notch pathway-dependent manner.
**CD11b+Jag2+ cells are mobilized to PB and home to tumor tissues in different colorectal cancer (CRC) models**

Considering the observed effect of BM-derived CD11b+Jag2+ cell population in inducing EMT in HCT15 cells *in vitro* and *in vivo*, next we quantified the recruitment of this population in other colon cancer models. For this purpose we developed subcutaneous colon carcinoma xenotransplants using 3 human CRC cell lines, HCT15, DLD-1 and HT-29, and evaluated the presence of CD11b+Jag2+ cells in the PB and in the tumor tissues. There was a significant increase in the frequency of CD11b+Jag2+ cells in the PB compared to control mice (no tumor) in all models (Figure 5A), this being particularly evident on HCT15 xenotransplanted mice, which showed a significant increase on days 7 and 14 after tumor inoculation. Moreover, FACS analysis of tumor samples showed that CD11b+Jag2+ cells are present in tumor xenotransplants derived from all cell lines at a frequency ranging from 4.5 – 8.5% of total cells (Figures 5B and C). To further validate the biological significance of CD11b+Jag2+ cells in CRC growth, we developed orthotopic models of CRC using HCT15 and HCT116 cell lines and evaluated their presence in the PB and in tumor tissues, as above. Mice bearing orthotopic HCT15 and HCT116 tumors showed increased frequency of PB CD11b+Jag2+ cells compared to control mice on weeks 4 and 6 following tumor inoculation (Figure 5D). Moreover, FACS analysis of tumor samples shows that CD11b+Jag2+ cells are present in orthotopic tumors at a frequency ranging from 0.5 – 6% of total tumor cells (Figure 5E and F). Together, these data shows that in different murine models of CRC, CD11b+Jag2+ cells are mobilized to the PB and are actively recruited into tumors, which is suggestive of the biological significance of this population in CRC growth and EMT onset.

**CD11b neutralizing antibodies reduce CD11b+Jag2+ cells recruitment, resulting in decreased tumor growth and EMT in vivo**

In order to further test the role of CD11b+Jag2+ in CRC progression and EMT, we treated mice bearing ectopic HCT15 tumors with neutralizing monoclonal antibodies against CD11b. CD11b neutralization caused a significant reduction in tumor growth (Figure 6A) and also on the PB levels of CD11b+ and CD11b+Jag2+ cells compared to mice treated with vehicle alone (Figure 6B). Moreover, flow
cytometry and immunostaining-based quantification of CD11b+ and CD11b+Jag2+ cells in tumor tissues showed that CD11b neutralization lead to a significant reduction in both cell populations (Figure 6C, D and E). Having shown that CD11b neutralization leads to a significant reduction in the number of PB and tumor-infiltrating CD11b+Jag2+ cells, we determined its effect in the onset of EMT. As shown in Fig 6, while control (untreated) mice show the expected decrease in E-cadherin expression (Figure 6F and G), this is sustained and even slightly increases in tumors of mice treated with CD11b neutralizing antibody. Analysis of Notch pathway downstream targets Hey 1 and Hes 1 expression showed the reduced EMT onset in tumors treated with anti-CD11b neutralizing antibody is accompanied by decreased activation of the Notch pathway (Figure 6F), which is in accordance with our *in vitro* data. Taken together, these data suggests that treatment of ectopic colon cancer-bearing mice with neutralizing monoclonal antibodies against CD11b reduces infiltration of CD11bJag2+ cells into the tumors, reducing EMT and reducing Notch pathway activation.

**CD11b+Jag2+ PB and tumor levels correlate with lower E-Cadherin expression and metastatic disease in colorectal cancer patients**

Having demonstrated the importance of BM-derived CD11b+Jag2+ cell population in inducing EMT in murine CRC models, and the easy quantification of these cells in PB of colon cancer bearing mice, next we tested the feasibility and usefulness of quantifying these cells in CRC patient samples. We quantified the levels of CD11b+Jag2+ cells in PB samples of 40 CRC patients diagnosed at different stages (according to the American Joint Committee on Cancer (AJCC) Staging System). For the analysis, patients were classified as: TxC0M0 (CRC patients without lymph node or distant metastasis, stages I-II of the AJCC), TxCxM0 (CRC patients with lymph node metastasis and without distant metastasis, stage III of the AJCC) and TxCxMx (CRC patients with distant metastasis, stage IV of the AJCC). We observed a significant correlation between PB CD11b+Jag2+ cell levels and CRC stages, with higher stage CRC patients showing higher PB CD11b+Jag2+ levels (Figure 7A and B). Moreover, there was an inverse correlation (*P* = 0.0197; *r²* = 0.4708, 11 patients) between PB CD11b+Jag2+ cell levels and tumor E-Cadherin expression (Figure 7C). We were also able to investigate the relation between CD11b+Jag2+ tumor levels and CRC staging in 12 patients. We observed
that TxNxM0 and TxNxMx stage patients had significantly higher numbers of CD11b+Jag2+ cells in tumor samples relatively to TxN0M0 patients (Figure 7D and E). We also observed a strong inverse correlation (P = 0.0007 ; r² = 0.7011, 12 patients) between CD11b+Jag2+ numbers in tumors and tumor E-Cadherin expression levels (Figure 7F). Importantly, in our patient cohort, there was no correlation between tumor size and the presence of PB or tumor CD11b+Jag2+ cells (Supplementary Fig 2). Together, these data strongly suggests that quantification of both CD11b+Jag2+ cells in the PB and in the tumor samples of CRC patients correlates with CRC staging, and thus may be used as a prognostic or diagnostic marker. These data also validate our data obtained from CRC mouse models, where we showed CD11b+Jag2+ cells infiltrate CRC tumors and induce EMT.

Discussion

Over the last decade there has been an increase in our understanding of the contribution of tumor stromal components in the regulation of tumor progression and metastasis formation(17). EMT has been shown to be essential for carcinoma progression, namely in breast, colon and prostate carcinoma, preceding invasion and metastasis formation(3, 18). One of the major molecular regulators of EMT is E-cadherin. E-cadherin is a member of the cadherin family of homophilic cell adhesion molecules, and is essential for the maintenance of adherens junctions that confer physical integrity and polarization to epithelial cells. Targeted disruption of E-cadherin during tumor progression resulting in decreased intercellular adhesiveness is one of the most common alterations in human cancers(19, 20). In fact, E-cadherin functional inactivation represents a critical step in the acquisition of invasive capacity by epithelial tumor cells. Accordingly, abolishing E-cadherin function in vitro confers invasive properties to noninvasive cells and conversely, introduction of E-cadherin into invasive epithelial cell lines abrogates their invasive potential. Not surprisingly, then, loss of E-cadherin expression is a defining feature of EMT (9). Although many molecular regulators of EMT have been identified the cellular interactions between tumor cells and tumor stromal cells responsible for tumor EMT are still unclear.
BM-derived cells, have been shown to directly impact tumor pathophysiology regulating multiple aspects of the metastization process by regulating tumor angiogenesis, promoting tumor cell invasion, extravasation, intravasation and micrometastasis establishment, growth and vascularization(21). However a role for BM-derived cells in the first steps of metastases formation, namely in EMT had not been addressed and was the subject of this study. In detail, we investigated a putative role for BM-derived cells in regulating EMT in metastatic colorectal cancer (CRC). We show in several ectopic and orthotopic CRC models that tumor progression is associated with an increased accumulation of BM-derived cells, mainly of myeloid origin. Furthermore we observe that in late tumors there are regions highly infiltrated by BM-derived cells that show evidence for EMT, namely a robust decrease in E-cadherin expression and a significant increase in vimentin expression in CK19+ tumor cells. It was highly suggestive from our analysis of serial tumor slides that tumor cells (CK19+) in close proximity with BM-derived cells (GFP+) showed the described hallmarks of EMT. This observation led us to speculate that a direct interaction between BM-derived and tumor cells could modulate EMT in the latter. To test this hypothesis, we used in vitro co-culture systems, where CRC cell lines are cultured with BM-derived tumor stromal cells (GFP+). We used this simple system to investigate the molecular regulation of EMT induction by BM-derived tumor associated cells. Although TGF-β pathway activation has been extensively described as an inducer of EMT(22), inhibition of TGF-β with neutralizing antibodies in our co-culture experiments, failed to inhibit the EMT-inducing capacity of BM-derived cells. On the other hand, the Notch pathway has also been associated with EMT and metastasis formation in pancreatic (23) and CRC (24). Moreover, Notch pathway activation was shown in breast cancer brain metastasis (25). Another study shows that in breast cancer patients, increased expression of either Jagged1 or Notch1 is predictive of poor overall survival(26). Moreover in colon cancer Notch 1 overexpression has been shown to correlate with pathologic grade, progression, and metastasis(27). Considering this we tested the effect of γ-secretase inhibitor (GSI, Notch pathway inhibitor) in reversing the EMT induction by BM-derived cells on CRC cell lines. GSI addition inhibited EMT induction by BM-derived cells, accompanied by a significant reduction in the expression of Notch pathway downstream targets Hey 1 and 2.
Although we did not address Notch downstream effectors regulating EMT, recent studies suggest the mechanisms by which Notch may induce EMT might involve activation of E-cadherin transcriptional repressor Snail2 (28, 29). Importantly, we show the BM-derived tumor infiltrating cells, which are mainly of myeloid origin (CD11b+) show increased expression of Jagged2, but not of other Notch ligands. Interestingly Jagged2 expression in tumor cells has been recently described as a major regulator of EMT and metastases in lung adenocarcinoma via a miR-200 dependent downstream mechanism (30). Furthermore, quantification of the GFP+ Jagged2+ populations present in the BM and in the tumors revealed a 10 fold increase in the percentage of this population in tumors, suggesting that BM-derived Jagged2+ cells are actively recruited into tumors. The signal(s) responsible for the selective recruitment of this specific BM-derived cell population into colon tumors remain undisclosed; we measured the most commonly studied chemokines including SDF1α, MCP1, but these were not actively secreted in our colon cancer models (not shown). Moreover, recent studies also showed that microparticles/microvesicles/exosomes released by tumors may communicate and “educate” the BM microenvironment resulting in selective recruitment of BM-derived cells to sites of metastases to form the pre-metastatic niche (31). Whether specific colon cancer-derived exosomes selectively induce the mobilization of CD11b+Jagged2+ cells is undisclosed. Concerning the molecular mechanisms regulating Jagged2 expression, recent data indicates its expression on breast cancer cells is regulated by hypoxia and is directly involved in breast cancer metastases (32). It is therefore legitimate to speculate that during CRC growth, recruited BM-derived CD11b+ cells enter the tumor site and are exposed to signals from the tumor microenvironment including hypoxia that regulate the expression of Jagged2. As shown here, once the infiltrating cells express Jagged2 these are capable of inducing EMT on subsets of colon cancer cells. The relative contribution of Jagged2 expressed by infiltrating BM-derived cells and by tumor cells proper for the onset of EMT is still undisclosed and will be the subject of future studies in our laboratory.

These findings led us to hypothesize that selective targeting of the CD11b+Jagged2+ BM-derived population might delay the onset of EMT, a first step in the metastasis cascade. Since we do not presently have access to specific
monoclonal neutralizing antibodies to Jagged2, we sought to impede the recruitment of CD11b+ cells (which include the Jagged2+ population) into the tumors. Several studies followed a similar approach, and concluded that targeting the CD11b+ population of infiltrating cells may be of therapeutic benefit (33, 34). Moreover, Toh et al (35) recently showed myeloid derived suppressor cells (also CD11b+) promote EMT, although they did not identify the mechanism involved. In our colon cancer models, CD11b neutralization lead to a significant decrease in CD11b+Jagged2+ cell PB and tumor levels, delayed EMT and reduced Notch pathway activation in the tumors. Together, these data strongly suggests that BM-derived CD11b+Jagged2+ cells represent a targetable cell population which is actively recruited into tumors and induces EMT in a Notch pathway dependent manner.

Having shown the biological significance of CD11b+Jagged2+ cells in mouse models of CRC we investigated the involvement of this cell population in human CRC progression. We observed that PB levels of CD11b+Jagged2+ cells correlate with CRC stages, with higher levels of CD11b+Jagged2+ cells being observed in CRC patients with metastatic disease (mostly consisting of liver metastases), while lower CD11b+Jagged2+ levels are detected in CRC patients without metastatic disease. Furthermore we found a significant correlation between CD11b+Jagged2+ cell PB levels and the levels of E-Cadherin expression in the corresponding (ie patient-specific) tumor tissues. Taking this into account we suggest the CD11b+Jagged2+ cells are biologically relevant in human CRC and that measuring PB levels of CD11b+Jagged2+ cells in CRC patients may be used as a biomarker to support CRC staging.

Our data highlight the importance of looking at the initial steps of the metastasis cascade also in a systemic manner. In detail, we show a BM-derived CD11b+Jagged2+ population can activate Notch signaling on colon cancer cells, resulting in EMT induction in vitro and in vivo. Given the recent excitement from clinical trials using Notch pathway inhibitors for the treatment of tumors, and particularly aiming at blocking tumor angiogenesis(36, 37), the data presented here paves the way for the use of Notch pathway inhibitors to impede metastases onset, by impeding EMT induction at a primary tumor site.
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References


Figure Legends

Figure 1. BM-derived cells infiltrate ectopic colorectal tumors and localize in regions undergoing EMT. 
A. Representative image of early (1 week) and late (3-4 weeks) sub-cutaneous colon tumors implanted into GFP+ BM nude mice. Scale bar: 100μm. Quantification of the GFP area percentage in both early and late tumors using ImageJ software. 
B. Representative image of GFP (green) and E-cadherin (red) expression on early and late colon tumors. Dashed line delimitates tumor regions that show decreased E-cadherin expression. Scale bar: 100μm. Quantification of the E-cadherin area percentage in both early and late tumors using ImageJ software. 
C. Representative image of GFP (green) and Vimentin (red) expression on early and late tumors. Scale bar: 100μm. Quantification of the number of vimentin + cells in both early and late tumors using ImageJ software. 
D. Correlation plot between E-cadherin area percentage and GFP area percentage (on the left) and also between the number of vimentin + cells and GFP area percentage (on the right). 
E. Representative image of sequential tumor sections of the same region stained for GFP and CK19, GFP and Vimentin, GFP and E-cadherin. Arrows point tumor cells (CK19+) in close contact with GFP cells that acquire Vimentin expression and lose E-cadherin expression. Scale bar: 10μm. Data are means ± s.e.m., * : p<0.05 ; n=7 (3 mice in early tumor group and 4 in late tumor group).

Figure 2. BM-derived cells induce EMT on colon carcinoma cells in a Notch pathway-dependent manner. 
A. Quantification of E-cadherin, Vimentin, Hey 1 and Hey 2 gene expression (normalized to 18s rRNA expression) by qRT-PCR on HCT15 alone (HCT15 C), in the presence of 10uM GSI (HCT15 + GSI), in the presence of BM-derived cells isolated from the tumors (HCT15 + TGFP) alone or in the presence of 10uM GSI (HCT15 + TGFP + GSI). 
B. Quantification of E-cadherin+ HCT15 cells on HCT15 C, HCT15 + GSI, HCT15 + TGFP and on HCT15 + TGFP + GSI co-culture conditions. Representative images of E-cadherin (red) expression in HCT15 cells co-cultured with BM-derived cells isolated from the tumors (GFP+; green). Data shown are means ± s.e.m of 3 independent experiments (n=3), * : p<0.05.

Figure 3. BM-derived cells expressing Jagged-2 induce EMT on colorectal cancer cells. 
A. Jagged-1, Jagged-2, Dll1 and Dll4 gene expression (normalized to 18s rRNA expression), determined by qRT-PCR in GFP+ cells collected from tumors or BM samples. Data shown are means ± s.e.m of 3 independent experiments (n=3), * : p<0.05 (between indicated groups), # : p<0.05 (between gene expression in BM or tumor samples). 
B. Quantification of the percentage of GFP+ Jagged-2+ cells in BM and tumor samples. Representative flow cytometry plot showing Jagged2+ BM-derived cells in tumor sample. 
C. Quantification of E-cadherin and (D) Hey 1 gene expression (normalized to 18s rRNA expression) by qRT-PCR on HCT15 alone (HCT15 C), in the presence of 10uM GSI (HCT15 + GSI), in the presence of tumor associated BM-derived Jagged-2+ cells (HCT15 + TGFP-Jag2+), in the presence of 10μM GSI and tumor associated BM-derived Jagged-2+ cells (HCT15 + TGFP+Jag2+ + GSI) or in the presence of tumor
associated BM-derived Jagged-2 negative cells (HCT15 + TGFP-Jag2-). E. Quantification of E-cadherin+ cells on HCT15 C, HCT15 + GSI, HCT15 + TGFP+Jag-2+, HCT15 + TGFP-Jag-2+ + GSI or HCT15 + TGFP-Jag-2- (negative) conditions. Panel shows representative images of E-cadherin (red) expression in HCT15 cells alone or in the different co-culture conditions. BM-derived Jagged-2+ cells isolated from the tumors are GFP+ (green). Data shown are means ± s.e.m of 3 independent experiments (n=3), * : p<0.05.

Figure 4. BM-derived CD11b+Jagged-2+ cells induce EMT via Jagged-2 mediated Notch pathway activation. A. Quantification of Jagged-2+ CD11b+/CD11b+ Gr-1+/F4/80+/Sca-1+/c-Kit+/CD3+/CD19+ expressing cells in tumors and corresponding BM samples. B. Quantification of the percentage of tumor infiltrating Jagged 2+ cells that express CD11b/CD11b Gr-1/F4/80/Sca-1/c-Kit/CD3 or CD19 marker. C. Representative flow cytometry plot showing CD11b+Jagged2+ BM-derived cells. D. Quantification of E-cadherin and Hey 1 gene expression (normalized to 18s rRNA expression) by qRT-PCR on HCT15 alone (HCT15 C), in the presence of 10uM GSI (HCT15 + GSI), in the presence of BM-derived CD11b+Jagged-2+ cells isolated from tumors (HCT15 + CD11b+Jag2+) and in the presence of 10uM GSI and tumor associated BM-derived CD11b+Jagged-2+ cells (HCT15 + CD11b+Jag2+ + GSI). E. Quantification of E-cadherin+ on HCT15 C, HCT15 + GSI, HCT15 + CD11b+Jag-2+ and HCT15 + CD11b+Jag-2+ + GSI groups. Representative images of E-cadherin (red) expression in HCT15 cells and CD11b (green) expression on tumor associated CD11b+ Jagged-2+ cells. Panel shows representative pictures of co-culture experiments where E-cadherin is labelled in red and CD11b BM-derived cells are labelled in green. Scale bar: 100um. Data shown are means ± s.e.m of 3 independent experiments (n=3), * : p<0.05.

Figure 5. CD11b+Jag2+ cells are mobilized to PB and home to tumor tissues in different colorectal cancer (CRC) models. A. Flow cytometry based quantification of CD11b+Jag-2+ percentage in PB mononuclear cell fraction of control (no tumor) and tumor bearing mice xenotransplanted with HCT15, DLD-1 and HT-29 colorectal cell lines, 7 and 14 days after ectopic tumor inoculation. B. Quantification of CD11b+Jag-2+ percentage in ectopic HCT15, DLD-1 and HT-29 tumor samples. C. Representative flow cytometry plot showing CD11b+Jagged2+ cells in ectopic HCT15, DLD-1 and HT-29 tumor samples. D. Quantification of CD11b+Jag-2+ percentage in PB mononuclear cell fraction of control and tumor bearing mice xenotransplanted with HCT15 and HCT116 colorectal cell lines, at week 2 to 10 (w2-w10) after orthotopic tumor inoculation. E. Quantification of CD11b+Jag-2+ percentage in orthotopic HCT15 and HCT116 tumor samples. F. Representative flow cytometry plot showing CD11b+Jagged2+ cells in orthotopic HCT15 and HCT116 tumor samples. Data are means ± s.e.m. , * : p<0.05 ; n=12 (3 mice per group in ectopic models) and n=12 (4 mice per group in orthotopic models).
Figure 6. CD11b neutralizing antibodies in vivo administration reduce CD11b+Jag2+ cells recruitment, resulting in decreased tumor growth and EMT. A. Ectopic tumor volume quantification in PBS (T) or anti-CD11b (T + a-CD11b) treated mice, 15 days after inoculation. Representative image of tumors collected at day 15 from control and anti-CD11b treated mice. B. Flow cytometry based quantification of the percentage of CD11b+ and CD11b+ Jag-2+ cells in the mononuclear cell fraction of PB samples of control (no tumor), PBS (T) and anti-CD11b (T + a-CD11b) treated mice, 15 days after inoculation. C. Flow cytometry based quantification of the percentage of CD11b+ and CD11b+ Jag-2+ cells in tumor samples of PBS (T) and anti-CD11b (T + a-CD11b) treated mice, 15 days after inoculation. D. Immunohistological quantification of CD11b+ cells in tumor samples of PBS (T) and anti-CD11b (T + a-CD11b) treated mice per 400x high power field (HPF), 15 days after inoculation. Representative images of CD11b+ cells (green) in T and T + aCD11b treated mice tumor samples. Scale bar: 50um. E. Immunohistological quantification of CD11b+ Jag-2+ cells in tumor samples of PBS (T) and anti-CD11b (T + a-CD11b) treated mice per 400x high power field (HPF), 15 days after inoculation. Representative images of CD11b+ (green) Jag-2+ (red) cells in T and T + aCD11b treated mice tumor samples. Scale bar: 50um. F. Quantification of E-cadherin, Hey 1 and Hes 1 gene expression (normalized to 18s rRNA expression) by qRT-PCR in tumor samples of PBS (T) and anti-CD11b (T + a-CD11b) treated mice. G. Quantification of the E-cadherin area percentage in tumor samples of PBS (T) and anti-CD11b (T + a-CD11b) treated mice. Representative images of E-Cadherin+ cells (red) in T and T + aCD11b treated mice tumor samples. Scale bar: 100um. Data are means ± s.e.m. , * : p<0.05 ; n=12 (4 mice per group: control, T and T + a CD11b).

Figure 7. CD11b+Jag2+ PB and tumor levels correlate with lower E-Cadherin expression and metastatic disease in colorectal cancer patients. A. Quantification of the number of CD11b+JAG2+ cells per ul of PB samles of CRC patients at different stages: TxN0M0 (CRC patients without lymph node or distant metastasis, stages I-II of the AJCC), TxNxM0 (CRC patients with lymph node metastasis and without distant metastasis, stage III of the AJCC) and TxNxMx (CRC patients with distant metastasis, stage IV of the AJCC). B. Representative flow cytometry plot showing CD11b+ Jagged2+ cells in PB samples of CRC patients at different stages. C. Correlation plot between the number of CD11b+JAG2+ cells in circulation and the E-cadherin score determined by immunostaining of primary tumor sections in individual CRC patient. D. Immunostaining quantification of CD11b+JAG2+ cells per 400x high power field (HPF) in primary tumor samples of CRC paients at different stages. E. Representative images of CD11b, Jag-2, E-cadherin and Cytokeratin-19 (CK-19) immunostainings in serial sections of primary tumor samples of CRC patients at different stages. F. Correlation plot between the number of tumor CD11b+JAG2+ cell number per 400x high power field (HPF) and the E-cadherin score determined by immunostaining of primary tumor sections in individual CRC patient. Data are indicated as means, * : p<0.05 using Mannn-Whitney test.
Figure 1
Figure 3
Figure 5
Figure 6
Bone marrow-derived CD11b+Jagged-2+ cells promote epithelial to mesenchymal transition and metastization in colorectal cancer

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