Inhibition of Dendritic Cell Maturation by the Tumor Microenvironment Correlates with the Survival of Colorectal Cancer Patients following Bevacizumab Treatment

Adriana J. Michielsen², Sinead Noonan², Petra Martin², Miriam Tosetto², Joseph Marry², Monika Biniecka¹, Aoife A. Maguire², John M. Hyland², Kieran D. Sheahan², Diamuid P. O’Donoghue³, Hugh E. Mulcahy², David Fennelly², Elizabeth J. Ryan², and Jacintha N. O’Sullivan²,³

Abstract

Development of bevacizumab has improved survival in colorectal cancer, however, currently there are no biomarkers that predict response to bevacizumab and it is unknown how it influences the immune system in colorectal cancer patients. Dendritic cells are important for the induction of an antitumor immune response; however tumors are capable of disabling dendritic cells and escaping immune surveillance. The aim of this study was to assess the numbers of CD11c+ cells infiltrating tumor tissue and to examine the effects of tumor conditioned media (TCM) and bevacizumab conditioned media (BCM) on dendritic cell maturation and correlate our findings with patient survival. colorectal cancer explant tissues were cultured with or without bevacizumab, to generate BCM and TCM, which were used to treat dendritic cells. CD80, CD86, CD83, CD54, HLA-DR, and CD1d expression was measured by flow cytometry. Interleukin (IL)-10 and IL-12p70 were measured by ELISA. The Cox proportional hazards model was used to associate survival with dendritic cell inhibition. TCM and BCM inhibited lipopolysaccharide (LPS)-induced dendritic cell maturation and IL-12p70 secretion (P < 0.0001), while increasing IL-10 secretion (P = 0.0033 and 0.0220, respectively). Inhibition of LPS-induced CD1d (P = 0.021, HR = 1.096) and CD83 (P = 0.017, HR = 1.083) by TCM and inhibition of CD1d (P = 0.037, HR = 1.036) and IL-12p70 (P = 0.037, HR = 1.036) by BCM was associated with poor survival in colorectal cancer patients. CD11c expression was elevated in tumor tissue compared with normal tissue (P < 0.001), but this did not correlate with survival. In conclusion, TCM and BCM inhibit dendritic cells, and this inhibition correlates with survival of colorectal cancer patients receiving bevacizumab. Mol Cancer Ther, 11(8); 1829–37. ©2012 AACR.

Introduction

Colorectal cancer is the third most common cancer in the world with the second highest mortality rate. About 25% to 30% of colorectal cancer patients present with metastases at diagnosis (1). Survival rates for these patients has significantly improved with the introduction of new targeted therapies, such as cetuximab (Erbitux) that targets the EGF receptor, and bevacizumab (Avastin), which is a humanized monoclonal antibody (mAb) against VEGF. However a response rate of 50% or less to these drugs has been reported, and in the case of bevacizumab, there are no current biomarkers that can predict response to this therapy (1, 2). For improved cost-effective treatment of metastatic colorectal cancer patients, discovery of biomarkers that can predict response to bevacizumab are warranted.

VEGF present in the tumor microenvironment drives angiogenesis and can also contribute to local immune evasion by the tumor (3–5). The local tumor environment of patients who fail to respond to bevacizumab likely produces high levels of other factors that effectively compensate for the lack of available VEGF, thus mediating angiogenesis and facilitating local immune suppression. We have previously shown that the local tumor microenvironment of metastatic colorectal cancer patients contains high levels of the chemokines CXCL1, CXCL5, and CCL2 that have proangiogenic properties. In addition we showed that conditioned media from colorectal cancer ex vivo explant tissue alters dendritic cell maturation and function (6). Dendritic cells are the most important antigen-presenting cells capable of activating naive T cells (7). Dendritic cells are present in tissues in an immature state and display low levels of maturation or costimulatory markers. Dendritic cells undergo a functional maturation

Authors' Affiliations:
¹Translation Rheumatology Research Group, Dublin Academic Medical Centre, The Conway Institute of Biomolecular and Biomedical Research, ²Centre for Colorectal Disease, Education and Research Centre, St. Vincent’s University Hospital, Elm Park, Dublin 4; and ³Institute of Molecular Medicine, Trinity Centre for Health Sciences, St James Hospital, Dublin 8, Ireland

Corresponding Author: Jacintha O’Sullivan, Institute of Molecular Medicine, Trinity Centre for Health Sciences, St James Hospital, Dublin 8, Ireland. Phone: 01-896-2149; Fax: 01-454-6534; E-mail: osullij4@tcd.ie
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process in response to inflammatory mediators such as IFN-α or Toll-like receptor agonists. As dendritic cells mature, they gain the potential of presenting antigen to T cells and activating a tumor antigen-specific T-cell response (8). Increased expression of several cell surface markers, such as CD54, CD80, CD86, CD83, and HLA-DR, on dendritic cells is associated with dendritic cell maturation and T-cell activation (9). Dendritic cells that secrete high levels of bioactive IL-12p70 induce optimal antitumor immunity, as they have increased capacity to enhance natural killer cell activity, skew the response to Th1, and prime tumor antigen-specific CD8⁺ T cells (10, 11). In addition, dendritic cells express the molecule CD1d, which presents lipid antigens and specifically activates natural killer T (NKT) cells, including invariant NKT cells (iNKT; ref. 12).

In this study, we explored the hypothesis that a patient’s lack of response to bevacizumab was because of a failure of this drug to neutralize the ability of the tumor microenvironment to suppress the function of dendritic cells. We investigated the levels of CD11c⁺ cells infiltrating into tumor using tissue microarrays (TMA). In addition, we used human explant colorectal cancer tissue to model the tumor microenvironment (13). We assessed the ability of tumor conditioned media (TCM) and bevacizumab conditioned media (BCM) from cultured human colorectal cancer explants to alter the function of dendritic cells and associate the effects with colorectal cancer patient survival.

Materials and Methods

Tissue microarray construction
A total of 85 colorectal cancer patients who received bevacizumab treatment (Genentech; 5–7.5 mg/m² every 2–3 weeks) at the Centre for Colorectal Disease, St. Vincent’s University Hospital, Dublin, Ireland, were enrolled for this study. A total of 23 consecutive patients undergoing colonoscopy for investigation of altered bowel habit and whose investigations were normal by both histologic and blood analysis were also included in this study as healthy controls (14). Table 1 shows the demographics of the colorectal cancer patients and healthy controls. Hematoxylin and eosin–stained slides from formalin fixed, paraffin-embedded tumor and nonadjacent normal tissue were used to identify specific areas, including the invasive margin of the tumor and normal adjacent tissue by a pathologist. These areas were aligned with the paraffin block from which four 6-mm cores were removed and transferred to a recipient block using a Tissue Microarrayer (Beecher Instruments). To account for heterogeneity in lymphocyte location in the tissues, we initially compared the staining of gross sections to the staining pattern of the TMAs for these patients, and we found that the TMA was representative of the gross sections for the immunohistochemistry markers we assessed in our study.

Immunohistochemistry
Immunohistochemistry was carried out as previously described (15). In short, 3-μm paraffin-embedded TMA sections were baked for 30 minutes at 90°C, deparaffinized in xylene, and rehydrated in alcohol and deionized water. Antigen retrieval was carried out by heating sections in antigen retrieval solution in a pressure cooker. Slides were washed in PBS and 0.05% Tween and non-specific binding was blocked using 10% casein in PBS. CD11c rabbit mAb (Abcam) was diluted 1:250 in antibody diluent (Dako) and incubated on sections for 1 hour at room temperature in a humidified chamber. An IgG control antibody and elimination of the primary antibody were used as negative controls. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide. Slides were washed in PBS and incubated with secondary antibody/horseradish peroxidase (Dako). DAB was used to visualize staining, 1:3 Mayer’s hematoxylin (BDH Laboratories) was used as a DNA counterstain. Stained sections were scanned using a Scan-Scope GL digital slide scanner and Aperios ImageScope software (Aperio Technologies). Expression of CD11c was assessed by 2 blinded reviewers scoring positive cell count in the stroma.

| Table 1. Patient demographics for CD11c expression |
|---------------------------------|-----------------|
| Cancer patients | Healthy controls |
| Male/female | 46/39 | 5/18 |
| Median age, y (range) | 64.4 (25.4–83.8) | 66 (56–84) |
| Stage of colorectal cancer at diagnosis |
| 1 Stage I | 14 Stage II |
| 32 Stage III | 38 Stage IV |
| Stage of colorectal cancer at start of bevacizumab treatment |
| 3 Stage III | 82 Stage IV |
| Median overall survival, d (range) | 554.0 (42.6–1,774.5) | — |
| Median duration of bevacizumab treatment, d (range) | 240.5 (12.2–1,263.2) | — |
Dendritic Cell Inhibition by Tumor Correlates with Survival

Ex vivo tumor explant culture

Surgically resected colorectal cancer tumor tissue was obtained from 22 patients from the Centre for Colorectal Disease’s explant tissue biobank at St. Vincent’s University Hospital, Dublin. Patient information is outlined in Table 2. All tissue was obtained with the written informed consent of the patient, and the study protocol was approved by the Ethics Committee at St. Vincent’s University Hospital. The explanted tissue was cut into 8 equal sized pieces of approximately 5 mm³ and cultured as previously described (6, 13). Briefly, the explanted tumor tissues were either untreated or treated with 100 µg/mL bevacizumab (Avastin) and cultured for 72 hours (in 24-well plates) in 2 mL RPMI-1640 containing 100 U/mL penicillin, 100 µg/mL streptomycin, 4 µg/mL fungizone, and supplemented with 10% FBS (Invitrogen). Following 72 hours in culture, TCM and BCM were collected and stored at −20°C until used for analyses.

Dendritic cell isolation and culture

Human monocyte-derived immature dendritic cells were generated from peripheral blood mononuclear cells obtained from buffy coat preparations (National Blood Centre, St. James’s Hospital, Dublin). Monocytes were isolated by positive selection using CD14 magnetic beads (Miltenyi Biotec) and seeded at a density of 1 × 10⁶ cells/mL in 6-well plates in 3 mL of RPMI-1640 medium containing 100 U/mL penicillin, 100 µg/mL streptomycin, 4 µg/mL fungizone, and supplemented with 10% defined Hyclone FBS (Thermo Fisher Scientific), human granulocyte macrophage colony-stimulating factor (50 ng/mL; Immunotools), and human IL-4 (70 ng/mL; Immunotools). Cells were fed at day 3 by replacing half the medium and adding fresh cytokines. At day 6, the cells exhibited an immature dendritic cell phenotype (CD14⁺, CD11c⁺, CD86⁺, CD54⁺/dim, CD83⁺, CD80⁺, and HLA-DR⁺/low).

Stimulation of monocyte-derived dendritic cells

Freshly isolated dendritic cells were plated in triplicate in 96-well plates at 1 × 10⁵ cells/200 µL in RPMI-1640 media supplemented with 10% defined Hyclone FBS (Thermo Fisher Scientific) and stimulated with a 1 in 2 dilution of TCM and BCM of all 22 patient explant tissues for 4 hours before adding 1 µg/mL Escherichia coli lipopolysaccharide (LPS; Alexis Biochemicals) for a further 18 hours. Supernatants were harvested and levels of IL-10 and IL-12p70 secretion analyzed by ELISA, and cells were assessed for expression of maturation markers by flow cytometry as described below.

Flow cytometry

Dendritic cells were stained with the following mAbs: fluorescein isothiocyanate–conjugated anti-CD14 and anti-CD80, phycoerythrin (PE)-conjugated anti-CD54 and anti-CD1d, PE-Cy5 (PeCy5)-conjugated anti-CD86 and anti-CD83, allophycocyanin (APC)-conjugated anti-CD11c and anti-HLA-DR (all from BD Biosciences). Cells were also stained with corresponding isotype control mAbs (BD Biosciences). Cells were acquired on FACScalibur flow cytometer, and the data were analyzed with CellQuest Pro (BD Biosciences) or FlowJo software (Tree Star Inc.).

Quantification of cytokines by ELISA

Levels of IL-10 and IL-12p70 in dendritic cell supernatant were quantified by sandwich ELISA according to the manufacturer’s protocol (R&D Systems).

Statistical analysis

Survival was calculated as time (days) from day of diagnosis until death of the patient. May 1st 2011 was used as cutoff time point for patients who survived until this date for the patients enrolled in the TMA study, whereas for patients enrolled in the explant study, 1st November 2010 was used as a cutoff time point.

Statistical analyses were carried out using GraphPad Prism version 5.00 for Windows (GraphPad software) and SPSS 15.00 for windows (IBM Ireland Limited). The paired student t test was used to compare groups. The Cox proportional hazards model was used to associate CD11c expression and dendritic cell inhibition with survival. A P value of less than 0.05 was considered to be significant. For the Kaplan–Meier curves patients were divided into 2 equal groups, divided by the median value of the marker measured (CD11c⁺, CD83⁺, CD1d⁺, IL-12p70⁻ and others). Kaplan–Meier curves were constructed for illustrative purposes only.

Results

Levels of CD11c⁺ cells are elevated in colorectal cancer patients compared with healthy controls

Numbers of CD11c⁺ cells infiltrating the tumor and adjacent normal tissue were assessed in 85 colorectal cancer cases and in 23 normal healthy controls using immunohistochemistry. Table 1 shows the characteristics of the patients and controls.

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<th>Table 2. Information on the patients whose explant tissue was used to generate TCM and BCM</th>
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<td>Male/female</td>
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<td>Median age, y (range)</td>
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<td>Stage of colorectal cancer at diagnosis</td>
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Figure 1A and B show representative staining of CD11c in colorectal tumor and adjacent normal tissue compared with age-matched healthy controls. Number of CD11c+ cells in the stroma of tumor and adjacent normal tissue of 85 colorectal cancer patients and 23 age-matched healthy controls were measured by immunohistochemistry. Scores of CD11c in the stroma of tumor and adjacent normal tissue of colorectal cancer patients were compared with scores in age-matched healthy controls. Representative images of low CD11c expression (A) and high CD11c expression (B) are shown. Numbers of CD11c+ cells in the stroma of tumor tissue (Fig. 1D) or adjacent normal tissue (data not shown) did not correlate with survival.

TCM and BCM alter dendritic cell maturation and IL-10 and IL-12p70 secretion

Surgically resected tumor tissue was obtained from 22 colorectal cancer patients at St. Vincent’s University Hospital, Dublin. Table 2 shows the patient information. Immature dendritic cells were preincubated with either TCM or BCM before LPS was added to induce dendritic cell maturation. Both TCM and BCM inhibited dendritic cell maturation in response to LPS: expression of CD80, CD54, CD86, HLA-DR, CD1d, and CD83 were reduced compared with cells treated with LPS alone (all P < 0.003 for both TCM and BCM; Fig. 2A). Figure 2B shows representative flow cytometry histograms of CD1d and CD83 expression by dendritic cells treated with LPS, TCM+LPS, and BCM+LPS. There was no difference between the effects of TCM and BCM on LPS-induced maturation of dendritic cells (Fig. 2A). In addition, we found that both TCM and BCM enhanced IL-10 secretion (TCM, P = 0.0033; BCM, P = 0.022) and decreased IL-12p70 secretion (P < 0.001 for TCM and BCM) induced by LPS (Fig. 2C). There was no difference between the effects of TCM and BCM on IL-10 and IL-12p70 secretion from LPS-stimulated dendritic cells (Fig. 2C). In summary, TCM and BCM inhibited dendritic cell maturation and IL-12p70 secretion, while enhancing IL-10 secretion in response to LPS.

Inhibition of CD1d and CD83 by TCM and BCM is associated with survival of colorectal cancer patients receiving bevacizumab

We associated survival of the 22 colorectal cancer patients with inhibition of dendritic cell maturation markers and IL-10 and IL-12p70 secretion induced by TCM and BCM using the Cox proportional hazards model (Table 3). Cox proportional hazards model associates the continuous data with survival. There is large variation in
survival rates between patients with a median survival of 1,134 days (range 217–2,938 days). Inhibition of LPS-induced CD1d and CD83 expression by TCM was significantly associated with an increased risk of death (CD1d: \( P = 0.021 \), HR = 1.096; CD83: \( P = 0.017 \), HR = 1.083; Table 3). The Kaplan–Meier curves of high versus low inhibition of CD1d and CD83 induced by TCM showed that patients with high inhibition of CD1d and CD83 have poorer survival compared with patients with low inhibition of CD1d and CD83 levels (Fig. 3A). Inhibition of CD1d and CD83 expression by BCM was also associated with poor survival (CD1d: \( P = 0.017 \), HR = 1.067; CD83: \( P = 0.032 \), HR = 1.035). In addition, inhibition of LPS-induced IL-12p70 secretion by BCM was significantly associated with poor survival (\( P = 0.037 \), HR = 1.036; Table 3). Corresponding Kaplan–Meier curves showed that high inhibition of CD1d, CD83, and IL-12p70 by BCM correlates with a higher risk of poor survival (Fig. 3B). Multivariate analysis showed that age, stage, and gender were not significantly associated with survival (data not shown).

**Discussion**

In this study, we examined the levels of infiltrating CD11c+ cells in colorectal cancer tissues of patients who...
received bevacizumab treatment. We found that numbers of CD11c+ cells were higher in tumor tissue compared with normal tissue from controls, although the frequency of CD11c+ cells infiltrating into the tumor did not correlate with patient survival. However, the activation state of dendritic cells is more important than dendritic cell number, as immature dendritic cells will induce tolerance whereas activated dendritic cells will induce immunity (16). We decided to examine the effect of conditioned media from cultured human colorectal cancer tumor tissue in the absence and presence of bevacizumab on the function of monocyte-derived dendritic cells. We found that both TCM and BCM significantly altered dendritic cell maturation, inhibiting the LPS-induced upregulation of several maturation markers. In parallel, cytokine secretion from dendritic cells treated with TCM-LPS and BCM-LPS compared with LPS alone was also altered; IL-10 secretion increased, which is an anti-inflammatory cytokine that inhibits a Th1 response (17), whereas IL-12p70 (a proinflammatory cytokine required for Th1 responses) secretions decreased: a dendritic cell phenotype associated with tolerance. In addition, we show for the first time that inhibition of LPS-induced expression of CD1d and CD83 by TCM and CD1d, CD83 and IL-12p70 by BCM is associated with poor survival in colorectal cancer patients. This illustrates the importance of understanding the immune regulation mechanisms occurring locally within the tumor microenvironment.

Few studies have examined CD11c+ cells in normal colon tissue; however, it has been reported in tumor tissues (18, 19). To our knowledge, this is the first study that shows that infiltrating CD11c+ cells in the tumor microenvironment are not associated with survival in colorectal cancer patients receiving bevacizumab treatment. However, dendritic cells expressing S100 or CD83 (a marker for dendritic cell maturation) have been associated with better survival in renal cell carcinoma and colorectal cancer, whereas the presence of immature CD1a+ dendritic cells in colorectal tumors correlates with recurrence of disease, indicating the importance of maturation of dendritic cells in predicting survival of colorectal cancer patients (20–23).

Several studies have shown that dendritic cell function and cytokine secretion were inhibited when dendritic cells were incubated with the commercial cell line supernatants of several different cancers including breast, colon, renal cell, and pancreatic carcinomas (3, 24–26). We have previously shown that conditioned media from explant tissue, which more accurately represents the tumor microenvironment including the different cell subtypes within the tumor, inhibit dendritic cell maturation and function (6). We found that TCM of explant tissues inhibit dendritic cell maturation by reducing the expression of CD80, CD54, CD86, HLA-DR, CD1d, and CD83, while also increasing IL-10 and decreasing IL-12p70 secretion. Furthermore, we investigated the effect of BCM on dendritic cells and found that BCM, like TCM, inhibited the LPS-induced expression of the same maturation markers and IL-12p70 secretion, while increasing IL-10 secretion. It has previously been shown that the addition of bevacizumab alone or in combination with sorafenib or sunitinib, which are tyrosine kinase inhibitors for the VEGF receptor, to dendritic cell cultures does not reverse the inhibitory effect of renal cell carcinoma (RCC-10) supernatants, indicating that VEGF is not the main mediator of dendritic cell inhibition in the RCC-10 supernatants (26). Due to the fact that many soluble factors are present in the tumor microenvironment, it is not surprising that inhibition of VEGF alone in BCM has no significantly different impact on dendritic cell maturation and function compared with TCM.

We examined whether inhibition of LPS induced dendritic cell maturation and cytokine secretion by TCM and BCM correlated with colorectal cancer patient survival. Inhibition of CD1d expression on dendritic cells treated under these conditions is associated with poor survival. CD1d is a molecule expressed on the surface of dendritic cells that presents lipid antigens to NKT cells and iNKT, which are tyrosine kinase inhibitors for the VEGF receptor, and activate the NKT cell receptor, to dendritic cell cultures does not reverse the inhibitory effect of renal cell carcinoma (RCC-10) supernatants, indicating that VEGF is not the main mediator of dendritic cell inhibition in the RCC-10 supernatants (26). Due to the fact that many soluble factors are present in the tumor microenvironment, it is not surprising that inhibition of VEGF alone in BCM has no significantly different impact on dendritic cell maturation and function compared with TCM.

We examined whether inhibition of LPS induced dendritic cell maturation and cytokine secretion by TCM and BCM correlated with colorectal cancer patient survival. Inhibition of CD1d expression on dendritic cells treated under these conditions is associated with poor survival. CD1d is a molecule expressed on the surface of dendritic cells that presents lipid antigens to NKT cells and iNKT. The correlation between CD1d and patient survival

| Table 3. Association between inhibition of dendritic cell maturation and cytokine secretion induced by TCM or BCM in response to LPS with survival of colorectal cancer patients |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Dendritic cell marker | P     | TCM HR (95% CI) | P     | BCM HR (95% CI) |
| CD80            | 0.578 | 0.989 (0.950–1.029) | 0.400 | 0.979 (0.931–1.029) |
| CD54            | 0.282 | 1.015 (0.988–1.042) | 0.224 | 1.018 (0.989–1.047) |
| CD86            | 0.829 | 1.004 (0.969–1.040) | 0.653 | 0.991 (0.955–1.029) |
| HLA-DR          | 0.163 | 0.971 (0.931–1.012) | 0.083 | 0.959 (0.914–1.005) |
| CD1d            | 0.021 | 1.096 (1.014–1.184) | 0.017 | 1.067 (1.012–1.124) |
| CD83            | 0.017 | 1.083 (1.014–1.157) | 0.032 | 1.035 (1.003–1.068) |
| IL-12p70        | 0.065 | 1.027 (0.998–1.056) | 0.037 | 1.036 (1.002–1.071) |
| IL-10           | 0.196 | 1.003 (0.998–1.008) | 0.163 | 1.003 (0.999–1.007) |

NOTE: Significant values are highlighted in bold. Statistics were carried out using the Cox proportional hazards model.
indicates that in addition to dendritic cells, NKT and iNKT cells may be important for colorectal cancer patient survival after treatment with bevacizumab. NKT cells play a major role in the initiation of a powerful antitumor response (27). Increased intratumor iNKT cells are a prognostic factor for primary colorectal tumors (28), whereas low levels of circulating iNKT cells predict poor clinical outcome in patients with head and neck squamous cell carcinoma (29). This indicates that iNKT may induce tumor cell death in patients with a good prognosis. Functional NKT cells were found in patients with chronic myelogenous leukemia, who had a complete cytogenetic response to imatinib (a reversible tyrosine kinase inhibitor), but not in patients with a partial response to imatinib (30). However the contribution of NKT and iNKT cells to patient survival after bevacizumab treatment has not been documented.

In addition, inhibition of LPS-induced CD83 expression by TCM and BCM is associated with poor prognosis. CD83 is predominantly expressed on mature dendritic cells and can enhance T-cell stimulation by dendritic cells (31). CD83+ cells infiltrating tumor tissue have been reported to have prognostic value in patients with breast carcinomas, gastric cancer, metastatic renal cell carcinoma, and colorectal liver metastasis (20, 21, 32, 33); however, the effect of the tumor microenvironment on CD83 expression on monocyte-derived dendritic cells has not been previously linked to cancer patient survival.
IL-12p70 is a proinflammatory cytokine, crucial for the induction of a potent antitumor response through multiple mechanisms. Studies have shown that increased levels of IL-12 induce antitumor immunity in animal models (34, 35) and that IL-12 may have additional antitumor effects by inhibiting angiogenesis and reducing MMP9 in experimental tumor models (35). We found that inhibition of IL-12p70 secretion from dendritic cells treated with BCM-LPS was associated with increased risk of death in colorectal cancer patients, indicating that IL-12p70 secretion from dendritic cells is an important factor for patient survival in metastatic colorectal cancer. We speculate that IL-12p70 could be used to assess treatment response to bevacizumab.

We observed no difference between TCM versus BCM on inhibition of LPS-induced expression of dendritic cell maturation markers and IL-10 and IL-12p70 secretion; however, the effect of BCM on IL-12p70 inhibition significantly correlated with patient survival, whereas TCM inhibition of IL-12p70 did not. An explanation for this could be the difference in inhibition of dendritic cell IL-12p70 secretion between TCM and BCM of individual patients.

In conclusion we found that TCM and BCM significantly inhibit dendritic cell maturation. Inhibition of LPS-induced CD1d and CD83 by TCM and BCM was significantly associated with an increased risk of death and inhibition of LPS-induced IL-12p70 by BCM also correlated with an increased risk of death. Altogether, this study shows that soluble factors secreted into the tumor microenvironment significantly inhibit dendritic cell maturation and cytokine secretion and that the addition of bevacizumab to the tissue culture does not reverse this inhibition, suggesting that factors other than VEGF present in the tumor microenvironment play an important role in the modulation of immune cells in the local tumor.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: A.J. Michielsen, D.P. O’Donoghue, D. Fennelly, E.J. Ryan, J.N. O’Sullivan
Development of methodology: J. Marry, D. Fennelly, J.N. O’Sullivan
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.J. Michielsen, S. Noonan, P. Martin, M. Tosetto, J. Marry, M. Biniecka, J.M. Hyland, K.D. Sheahan, D.P. O’Donoghue
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.J. Michielsen, S. Noonan, M. Biniecka, H.E. Mulcahy, E.J. Ryan, J.N. O’Sullivan
Writing, review, and/or revision of the manuscript: A.J. Michielsen, S. Noonan, P. Martin, A.A. Maguire, K.D. Sheahan, H.E. Mulcahy, D. Fennelly, E.J. Ryan, J.N. O’Sullivan
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Noonan, P. Martin, M. Tosetto, J.M. Hyland, K.D. Sheahan, D. Fennelly
Study supervision: D.P. O’Donoghue, H.E. Mulcahy, D. Fennelly, J.N. O’Sullivan

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References


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