Tumour microenvironment of both early- and late-stage colorectal cancer is equally immunosuppressive

A O’Toole1,2, A J Michielsen1,3, B Nolan1, M Tosetto1, K Sheahan1,2, H E Mulcahy1,2, D C Winter1,2, J M Hyland1,2, P R O’Connell1,2, D Fennelly1,2, D O’Donoghue1,2, J O’Sullivan3, G A Doherty1,2 and E J Ryan *,1,2

1Centre for Colorectal Disease, St Vincent’s University Hospital, Dublin 4, Ireland; 2School of Medicine and Medical Sciences, University College Dublin, Dublin 4, Ireland and 3Department of Surgery, Institute of Molecular Medicine, Trinity Centre for Health Sciences, St James’s Hospital, Dublin 8, Ireland

**Background** Tumour microenvironment (TME) of advanced colorectal cancer (CRC) suppresses dendritic cell (DC) maturation. Here, our aim was to determine how the microenvironment of early-stage tumours influences DCs.

**Methods:** Tumour-conditioned media (TCM) was generated by culturing explant tumour tissue in vitro (n = 50). Monocyte-derived DCs (MDDCs) of healthy donors or cancer patients were pretreated with TCM and stimulated with lipopolysaccharide (LPS). DC maturation was assessed by flow cytometry and cytokine production measured by ELISA.

**Results:** TCM from both early- and late-staged tumours abrogated LPS-induction of IL-12p70 secretion, while increasing IL-10. The profile of inflammatory mediators in TCM was similar across stages, and all increased pSTAT3 expression by DCs. CRC patient DCs (n = 31) secreted low levels of IL-12p70 and failed to upregulate expression of maturation markers in response to LPS. Furthermore, in vitro culture of autologous DCs with TCM did not change the hypo-responsiveness of patient DCs.

**Conclusion:** Our data demonstrates that the TME of all stages of CRC contains inflammatory mediators capable of suppressing local DCs. MDDCs obtained from CRC patients are hyporesponsive to stimuli such as LPS. Measures to reverse the negative influence of the TME on DCs will optimise cancer vaccines in both early- and late-stage CRC.

Chronic inflammation in the tumour microenvironment (TME) is integral to the neoplastic process; enhancing tumour growth, invasion, angiogenesis and metastasis (Lin and Karin, 2007). Dendritic cells (DCs) are a key player in orchestrating the immune response in the TME (Palucka and Banchereau, 2012). We have previously demonstrated that the TME of advanced colorectal cancer (CRC) suppresses monocyte-derived dendritic cell (MDDC) maturation and IL-12p70 secretion (Michielsen et al, 2011). Importantly, the degree of this inhibition correlated with the survival of stage IV patients receiving bevacizumab therapy (Michielsen et al, 2012). Although changes in DC phenotype have been noted in both colorectal (Gulubova et al, 2012) and ovarian (Scarlett et al, 2012) cancer progression, it is not clear if the immunosuppressive nature of the TME varies with disease progression in colorectal cancer.

In a p53-dependant model of ovarian carcinoma depletion of DC in early stages of disease accelerated tumour expansion; but at later stages of disease significantly delayed malignant progression (Scarlett et al, 2012). This suggests a bi-modal role for DCs in cancer progression. However, it is not known if or when a switch in the microenvironmental regulation of DC function occurs in CRC progression (Seton-Rogers, 2012). A clearer understanding of how the suppressive nature of the tumour microenvironment influences the function of DCs in different cancer patient cohorts would...
facilitate development of more effective immunotherapy and DC-based cancer vaccines.

Here we used our established tumour explant model (Michielsen et al, 2011, 2012) to determine if the microenvironment of earlier staged tumours is as suppressive as that of patients with metastatic disease. In addition we characterised the phenotype of non-metastatic CRC patients’ MDDCs and examined production of IL-12p70 in response to lipopolysaccharide (LPS) in both the absence and presence of tumour-conditioned media.

MATERIALS AND METHODS

Ex vivo tumour explant culture. Surgically resected tumour tissue was collected from patients with CRC (n = 50). Patient characteristics and details of tumour pathology are presented in Table 1. Sixty four percent of the cohort was male. Median age at diagnosis was 67.4 years (range, 32–93). Tumour-conditioned media (TCM) was prepared as previously described (Michielsen et al, 2011). Briefly, the explanted tissue was cut into four equal-sized pieces of approximately 5 mm3 and cultured (in 24-well plates) in 2 ml Briefly, the explanted tissue was cut into four equal-sized pieces of

Table 1. Characteristics of the cohort of colorectal cancer (CRC) patients and summary of the pathology of the tumours used to generate tumour-conditioned media (n = 50)

|                | Stage I | Stage II | Stage III | Stage IV |
|----------------|---------|----------|-----------|----------|
| N              | 8       | 21       | 14        | 7        |
| Male/Female    | 6/2     | 15/6     | 9/5       | 2/5      |
| Age, median (range) | 68 (46–80) | 73 (53–84) | 65 (32–93) | 66 (58–82) |
| CRC mortality  | 1       | 4        | 4         | 1        |
| Lymphovascular invasion | 0       | 9        | 11        | 6        |
| Perineural infiltration | 0       | 0        | 4         | 2        |
| Mismatch repair loss | 1       | 2        | 3         | 0        |
| KRAS mutation  | Not tested | Not tested | 4        | 6        |
| Tumour budding  | 1       | 0        | 3         | 3        |

Namely C0 was prepared as previously described (Michielsen et al, 2011). Tumour-conditioned media (TCM) was collected from patients with CRC (2011). Tumour-conditioned media (TCM) was generated

Cytokine analysis. Cytokines were quantified by ELISA using the manufacturer’s protocol; IL-10, IL-12p70 (R&D Systems, Oxford, UK) and IL-6 (Biologend, San Diego, CA, USA). The detection limits of the assays were as follows; IL-10, 31.2 pg ml−1; IL-12p70, 31.2 pg ml−1 and IL-6, 7.8 pg ml−1.

TUM from all stages of CRC was screened for the following cytokines: Angiogenin, EGF, ENA-78, bFGF, BIO, IFN-γ, IGF-1, IL-6, IL-8, Leptin, MCP-1, PDGF-BB, PIGF, RANTES, TGFβ1, TIMP1, Thrombopoietin, VEGF and VEGF-D using a Cytokine Antibody Array (RayBiotech, Inc, Norcross, GA, USA) according to the manufacturer’s protocol.

Phospho-STAT3 analysis. Immature MDDCs (1 x 106 cells per ml) were incubated at 37 °C, 5% CO2 for 2 h, and then treated with either recombinant IL-6 (R&D Systems) at 20 ng ml−1 or a 1:2 dilution of TCM for 15 min. Cells were fixed with Cytofix buffer (BD Biosciences), permeabilized with Phosflow Perm Buffer III (BD Biosciences) and pSTAT3 expression was measured using phospho-STAT3 (BD Biosciences) following the recommended protocol. Samples were acquired on a FACScalibur flow cytometer.

Statistical analysis. Statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad software, La Jolla, CA, USA). ANOVA with the Kruskal–Wallis post hoc test was used to compare multiple groups of nonparametric data, whereas the Mann–Whitney U-test to compare two groups of unpaired data. P-values < 0.05 were considered significant in all the analyses.

RESULTS

TME of all stages of colorectal cancer modulates MDDC response to LPS. Tumour-conditioned media (TCM) was generated from CRC tumours with different stages of disease progression (stage I, n = 8; stage II, n = 21; stage III, n = 14, stage IV, n = 7). Tumours were assigned as stage I–IV according to the American Joint Committee on Cancer (AJCC) classification system (Edge and Compton, 2010). We found that TCM from all stages of CRC significantly suppressed IL-12p70, whereas levels of IL-10 were increased in response to LPS (Figure 1A). TCM pretreatment of MDDCs also suppressed the upregulation of maturation markers in response to LPS (Figure 1B and C). Immature MDDCs were cultured with TCM without the addition of LPS as a control, however this had no effect on cytokine secretion or maturation marker expression when compared with immature DCs cultured in media alone (data not shown). These data clearly illustrate that the microenvironment of both early and advanced colorectal cancer has the potential to potentely suppress cell-mediated immunity.

Profile of inflammatory mediators in both the early and advanced colorectal cancer microenvironment. TCM generated from CRC stages I–IV were screened for the presence of a panel of inflammatory mediators using an antibody based protein-array. Figure 2A shows that TCM of all stages of CRC progression contains a similar profile of inflammatory mediators. Of note, high levels of IL-6, IL-8, GRO and angiogenin are present. Using ELISA we confirmed that IL-6 was present in equivalent concentration in TCM from all tumour stages (Figure 2B).

Next we determined if TCM of colorectal tumours equally induce STAT3 phosphorylation in DCs. Briefly, MDDCs were incubated for 15 min with a 1:2 dilution of TCM from stage I–IV...
CRC (n = 5, per stage) or recombinant IL-6 (positive control), and levels of pSTAT3 measured by flow cytometry. We found that four of the five patients’ TCM of each stage I–III induced significant upregulation of pSTAT3 expression by MDDCs, however TCM from five of the five cases tested with stage IV disease TCM induced pSTAT3 expression (Figure 2C).

MDDCs of stage II colorectal cancer patients are hypo-responsive to LPS. Next, to determine if the local microenvironment of the tumour could modulate the function of autologous DCs, we cultured MDDC from presurgery blood samples in the presence of TCM generated from the patient’s own tumour (n = 13, stage I, n = 1, stage II, n = 11, stage III, n = 1). Median age of the cohort was 60.8 years (range, 50–84). Nine patients were male. Further clinical details of this cohort are presented in Table 2. TCM used in these experiments suppressed the responsiveness of healthy control MDDCs to LPS as we have previously observed (Figure 3). However, as the response of the patient MDDCs to LPS was poor, TCM pretreatment of patient DC did not have an additional inhibitory effect. Of particular note, MDDCs obtained from this
cohort of patients with early-staged CRC secreted very low levels of IL-12p70 in response to LPS. Although pretreatment of patient or control MDDCs with TCM increased IL-10 production in response to LPS, this did not reach statistical significance in these experiments.

In addition, MDDCs were obtained from a further \( n = 31 \) patients with CRC before surgery. Clinical and pathological details of this cohort are presented in Table 2. (stage I, \( n = 6 \); stage II, \( n = 15 \); stage III, \( n = 9 \); stage IV, \( n = 1 \), with a median age at diagnosis 63 years (44–82)). Interestingly, we found that in general the patients’ MDDCs secreted low levels of IL-12p70 in response to LPS (median, 39.8 pg ml\(^{-1}\), (6.9–990 pg ml\(^{-1}\)).

**DISCUSSION**

Tumour-derived factors drive the evolution of an immuno-suppressive network, thus contributing to tumour invasiveness and metastasis (Kim et al, 2006; Gabrilovich et al, 2012). A deeper understanding of this process in humans is urgently needed to optimise immunotherapeutic strategies. In this study, using tumour tissue explants we confirm that the TME of all stages of colorectal cancer exerts a significant suppressive effect on DCs. Previous work has suggested that colorectal cancer patients have both numerical and functional impairment in peripheral blood (Della Porta et al, 2005) and local tumour-infiltrating DCs (Schwaab et al, 2001; Dadabayev et al, 2004; Inoue et al, 2005; Cui et al, 2007; Yuan et al, 2008). We found that MDDCs obtained from CRC patients with non-metastatic disease were hyporesponsive to LPS, secreting markedly reduced levels of IL-12p70 compared with MDDCs obtained from healthy controls. Decreased IL-12B transcription in monocytes obtained from patients with CRC was previously reported to be associated with the stage of CRC (Stanilov et al, 2012). Yet, in our study, MDDCs of those patients with non-metastatic disease (stage I and II) were just as nonresponsive to LPS as those with metastatic disease. Data from a recently reported clinical study indicate the absolute requirement...
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for IL-12p70 secretion for the efficacy of DC therapies (Carreno et al., 2013), highlighting the need to understand how IL-12p70 is regulated in the TME. Our finding that the TME of all CRC tumours tested (n = 50) potently suppressed IL-12p70 secretion may also help explain why often DC vaccines that are capable of inducing T-cell responses in vitro may fail in vivo (Rossowska et al., 2009).

Local activation of NF-κB and resultant inflammatory cytokines are of great importance in the shaping of the local tumour environment (Muthuswamy et al., 2012). We screened the TME of both metastatic and non-metastatic tumours to determine if there were different patterns of local inflammation and found high levels of inflammatory mediators, notably IL-6 and IL-8, in all the samples. IL-8 can contribute to colon carcinogenesis by increasing local infiltration and retention of immature myeloid cells (Feijoo et al., 2005; Asfaha et al., 2013). IL-6 also promotes accumulation of myeloid derived suppressor cells (MDDCs; Bunt et al., 2007). In addition, IL-6-mediates arginase activation and the subsequent reduction in MHC class II expression on DC, a mechanism critical for inducing dysfunction of the immune system in the tumour-reduction in MHC class II expression on DC, a mechanism critical in mediating the influence of the TME on DC differentiation and function (Nefedova et al., 2004). We found TME of both metastatic and non-metastatic tumours upregulated pSTAT3 expression in MDDCs. Interestingly, STAT3-depleted human DCs with adenoviral STAT3 short hairpin RNA were capable of producing more cytokines with TLR stimulation and were resistant to cancer-derived factors, inducing tumour Ag-specific T cells more efficiently than control DCs (Iwata-Kajihara et al., 2011).

To conclude we have demonstrated that MDDCs of non-metastatic CRC patients secrete low levels of IL-12p70 in response to LPS and that the TME of CRC of all stages of disease progression is able to suppress the function of MDDCs. These data add some strength to the argument in favour of some form of preoperative immune boosting strategy to increase disease-free survival following surgical resection (Lonnroth et al., 2008).

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