

Enrichment of inflammatory IL-17 and TNF- α secreting CD4⁺ T cells within colorectal tumours despite the presence of elevated CD39⁺ T regulatory cells and increased expression of the immune checkpoint molecule, PD-1

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Enrichment of inflammatory IL-17 and TNF- α secreting CD4⁺ T cells within colorectal tumours despite the presence of elevated CD39⁺ T regulatory cells and increased expression of the immune checkpoint molecule, PD-1

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Abbreviations used:

CRC = Colorectal cancer

PBMC = Peripheral blood mononuclear cells

FBS = Foetal bovine serum

DMSO = Dimethyl sulfoxide

PBS = Phosphate buffered saline

PenStrep = Penicillin Streptomycin

PMA = Phorbol myristate acetate

Treg cell = Regulatory T cell

CTLA-4 = Cytotoxic T lymphocyte antigen 4

PD-1 = Programmed cell death 1

IL- = Interleukin

TNM = Tumour node metastasis

LVI = Lymphovascular invasion

EMVI = Extramural vascular invasion

NI = Neural Invasion

Abstract

T cell infiltration into colorectal tumours has been shown to correlate with improved patient outcomes. However, more detailed information on the make-up and relationships between the infiltrating T cell subsets is lacking. We therefore correlated the extent of immune infiltration into colorectal tumours with the frequencies of various T cell subsets. We prospectively recruited 22 patients at the time of surgical resection for colorectal cancer. The Klintrup–Mäkinen (KM) score was used to estimate the extent of immune infiltration into colorectal tumours. The frequencies of CD4 and CD8 T cells that produced cytokines or expressed the inhibitory molecule PD-1 were determined by flow cytometry in colorectal tumour and matched uninvolved colonic tissue. In addition, the frequency of CD4 regulatory T cell subsets was determined. An increased frequency of CD4 T cells producing IL-17 (Th17 cells) was observed in colorectal tumour tissue compared with adjacent uninvolved tissue. These Th17 cells mostly co-produced TNF- α but not IFN- γ . IL-17 expression correlated positively with TNF- α and IL-10. Increased expression of the immune checkpoint molecule PD-1 was found in colorectal tumours compared with adjacent uninvolved tissue. There was a negative correlation between expression of PD-1 and IFN- γ but not IL-17, for both CD4⁺ and CD8⁺ T cells. CD4⁺CD25⁺CD127^{lo} and CD4⁺CD25⁺CD127^{lo}FoxP3⁺CD39⁺ Treg cells were enriched in colorectal tumours.

A positive correlation between KM score and percentage CD4⁺CD25⁺CD127^{lo} Treg cells was observed in tumours suggesting that increased immune infiltration is associated with an increased proportion of Treg cells. In addition, there was a negative correlation between the frequency of CD4⁺CD25⁺CD127^{lo} Treg cells and the expression of IFN- γ and IL-2, but not IL-17, in tumours. Taken together these data suggest that both PD-1 expressing T cells and Treg cells within the tumour may have a suppressive effect on T cells secreting IFN- γ , IL-2 or TNF- α but not Th17 cells.

1. Introduction

There is accumulating evidence indicating that the number, type and location of tumour infiltrating lymphocytes has prognostic value in colorectal cancer (CRC); where robust T cell infiltration correlates with improved outcome (1, 2). These data have led to the development of the immunoscore, derived from measurement of memory (CD45RO) CD3 and CD8 T cell infiltration into the tumour centre and invasive margin. Significantly, the immunoscore correlated positively with improved outcome regardless of stage and is being validated in large scale studies (3).

However tumour infiltrating T cells contain a number of different functional subtypes which can have either pro- or anti-tumour effects. Cytotoxic IFN- γ producing CD8 T cells play a key role in the anti-tumour response. The role of CD4 T cells, which can be divided into Th1, Th2, Th17 and Treg cell subsets is more complex. Th1 cells, which produce IFN- γ and provide help to CD8 T cells, are considered to have an anti-tumour role (4). However the role of Th17 cells in tumour immunity remains controversial. In the context of autoimmunity Th17 cells are pro-inflammatory and pathogenic. While studies in murine cancer models indicate an anti-tumour role for Th17 cells (5-7), there is contrasting evidence from other murine and human studies suggesting that Th17 cells promote angiogenesis and drive tumour development (4, 8, 9). IL-17 promotes angiogenesis by inducing VEGF production by tumour cells (9) and can mediate resistance to anti-VEGF therapy in murine models (10). Furthermore the tumour microenvironment promotes the recruitment and expansion of human Th17 cells (11). Importantly, patients with low expression of Th17 related genes exhibited prolonged disease free survival (4). Thus on balance, the data from human studies appears to favour a model where Th17 cells promote angiogenesis and tumour development.

Treg cells play a crucial regulatory role in maintaining tolerance and preventing autoimmunity. However, in the context of cancer, the general consensus is that Treg cells inhibit anti-tumour responses and contribute to the immunosuppressive microenvironment. However it is possible that Treg cells could play a dual role by initially dampening pro-tumour inflammation, but later acting to inhibit anti-tumour effector cells in the established tumour. In CRC Treg cells have been shown to be enriched in the tumour (12), however the role of these Treg cells remains controversial (13). In contrast to findings in other cancer settings, high levels of CRC tumour infiltrating Treg cells were associated with early stage disease and improved prognosis (14, 15). Other studies however did not find a positive correlation between good prognosis and Treg cell infiltration (16, 17). The question of how tumour infiltrating Treg cells regulate local effector T cells within the tumour microenvironment remains to be determined.

The introduction of cancer immunotherapies including those targeting CTLA-4 and PD-1/PD-L1, have highlighted the therapeutic relevance of understanding the regulation of local tumour immunity (18, 19). However, the success of immunotherapy is variable between patients and likely to be dependent on the presence of the relevant target; for example the success of targeting the PD-1/PD-L1 axis was limited to patients with tumours expressing PD-L1 and with CD8 T cell infiltration (20) and so far these drugs have demonstrated poor clinical efficacy in CRC (21). Here, we report a detailed analysis of tumour infiltrating Treg cells and PD-1⁺ lymphocytes in parallel with documenting the cytokine producing potential of effector cells within the same tumour. Interestingly, we found that despite being highly infiltrated by potentially immunosuppressive CD39⁺ Treg cells, colorectal tumours contain significant numbers of pro-inflammatory IL-17 and TNF- α secreting T cells. These effector T

121 cells are likely to be a key component of the local tumour-sustaining inflammatory
122 environment that is increasingly being recognised as a characteristic of solid tumours (22).

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2. Materials and Methods

2.1 Patient samples

Colorectal tumour and adjacent histologically normal tissue samples (at least 10 cm from tumour site) were obtained from 22 patients undergoing surgical resection at St. Vincent's University Hospital, Dublin. Patient demographics and clinical details are reported in Table 1. Informed consent was obtained from all participants. Ethical approval for this study was granted by the St Vincent's Healthcare Group Ethics and Medical Research Committee and research was conducted according to the Helsinki guidelines.

2.2 Histopathological analysis

The samples from surgical specimens were fixed in 10% buffered formalin solution and embedded in paraffin, and 5 mm sections were stained with H&E. We evaluated peritumoural inflammatory reaction from H&E slides according to the K-M criteria, where 0 denoted no recruitment of inflammatory cells, 1 denoted mild and patchy increase of inflammatory cells, 2 denoted a band-like infiltrate at the invasive margin with some evidence of destruction of cancer cell islets, and 3 denoted a very prominent inflammatory reaction with frequent destruction of cancer cells. These scores were then classified into low grade (0–1) and high grade (2–3) (23, 24). All cases were also evaluated for the absence or presence of Crohn's-like reaction (CLR).

2.3 Cell isolation

Tissue samples were collected and transported in Hank's Balanced Saline Solution, calcium- and magnesium-free (HBSS, Sigma-Aldrich), supplemented with 1% FBS (Sigma) and Penstrep, Gentamycin and Fungizone (Labtech (Life Technologies)). Tissue fragments were washed thoroughly, chopped and then digested in 125 U/ml collagenase type IV-S, sterile filtered, (Sigma-Aldrich) in HBSS. Tissue was incubated at 37°C with agitation, for no longer than 30 min. Cells were then filtered through a 70 nm nylon mesh (BD Biosciences), washed in warm HBSS and counted. Cells were either stained immediately for Treg cell markers and T cell memory markers, or incubated overnight in complete RPMI medium (supplemented with 10% FBS and Penstrep) prior to restimulation with PMA (10 ng/ml) and ionomycin (1 µg/ml) in the presence of brefeldin A (5 µg/ml) (all Sigma-Aldrich) for 3.5 hours.

2.4 Flow cytometry staining

For analysis of PD-1, cells were washed in serum-free PBS and stained with a fixable viability dye eFluor506, CD4-PerCPCy5.5, PD1-PECY7, CD8-APCeFluor780, (eBioscience), incubated for 15 min at room temperature in the dark, then washed in PBS buffer containing 1% FBS and sodium azide. Cells were stained for Treg cell markers using a FoxP3 staining buffer set (eBioscience) and accompanying protocol. Treg cell markers included CD39-FITC, FoxP3-PE, CD73-PerCPeFluor710, CD25-PECY7, CTLA-4-APC, CD127-APCeFluor780, Ki67-eFluor450 (eBioscience) and CD4-V500 (BD Biosciences). An intracellular staining kit (Fix and Perm kit, Invitrogen) was used to analyse cytokine production after restimulation with PMA/ionomycin. Cells were stained with IL-17A-AlexaFluor488, IL-10-PE, TNF-α-PerCPCy5.5, CD45RA-PECY7, CD8-APCeFluor780, FoxP3-eFluor450, (all eBioSciences), CD45 AlexaFluor700 (BioLegend), IFN-γ-APC, CD3-V500, and IL-2-PE-CF594 (BD Biosciences). Due to PMA/ionomycin-mediated reduction in CD4 expression, CD4⁺ T cells were identified as CD3⁺CD8⁻ T cells for cytokine analysis. Cells were acquired on a BD LSRFortessa flow cytometer, and analysed using FlowJo software (FlowJo LLC).

2.5 Statistical analysis

Analysis of statistical differences in T cell population frequency and cytokine production between paired uninvolved tissue and tumour samples was performed using Prism GraphPad version 6. All data was assumed to be non-parametric and was analysed using the Wilcoxon matched pairs test. Pearson's correlation coefficient (r) was calculated using the Statistical Package for the Social Sciences (SPSS) version 18.0, IBM.

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3. Results

3.1 Varied grades of immune cell infiltration into colorectal tumour tissue

We prospectively recruited 22 patients at the time of surgical resection for colorectal cancer. Details of the patient cohort are shown in Table 1. All patients were treatment naïve at the time of surgery. Mismatch repair (MMR) status for all cases was determined by immunohistochemistry for the mismatch repair proteins, MLH1, PMS2, MSH2 and MSH6. None of the tumours included in our analyses displayed Microsatellite instability (MSI).

In this study we used the Klintrup–Mäkinen (KM) score to estimate the extent of immune infiltration into colorectal tumours. After surgical resection, colorectal tumour tissue samples were paraffin embedded, sectioned and H&E stained. Representative examples of KM scores 1-3 are shown in Figure 1 A-C. Of the 22 cases examined only n=2 had a KM score of 0, indicating that the majority of MSS tumours included in this study were infiltrated by immune cells. We also documented the absence or presence of a Crohn's like reaction in all patient samples. An example of Crohn's like staining is shown in Figure 1D. Of the 22 tumours examined 15 displayed evidence of a Crohn's like reaction, again indicating a significant degree of immune cell infiltration in the tumours of this cohort.

In addition, histologically normal adjacent tissue and tumour tissue was finely chopped and enzymatically digested to obtain single cell suspensions that were analysed by flow cytometry to assess the phenotype of tumour infiltrating T cells. Cells were stained with antibodies specific for CD45 to identify tumour infiltrating leukocytes and panels of antibodies specific for T cell markers to identify specific subsets of T lymphocytes. Compared to uninvolved adjacent tissue, colorectal tumour tissue had an increased frequency of total T cells ($p<0.05$) ($CD3^+$ lymphocytes, Fig 1E). However, both the uninvolved normal tissue and colorectal tumour tissue were infiltrated by $CD4^+$ and $CD8^+$ T cells, with a CD4:CD8 ratio of approximately 2:1 (Figure 1F-G). The vast majority of T cells infiltrating tumours were found to be of the effector memory type (data not shown). However within the immune infiltrate, the ratio of $CD4^+$ to $CD8^+$ T cells did not differ from uninvolved adjacent tissue.

3.2 Increased frequency of $CD4^+IL-17^+$ (Th17) cells infiltrating colorectal tumours

We then aimed to further dissect the phenotype and function of these colorectal tumour infiltrating lymphocytes by analysing cytokine production by individual T cell subsets using intracellular staining and flow cytometry. Single cell suspensions from normal adjacent tissue and colorectal tumour tissue were prepared from fresh surgical explants and stimulated for 3.5 hours with PMA and ionomycin in the presence of brefeldin A. Cells were then stained with antibodies specific for T cell markers, permeabilised and incubated with antibodies specific for the following cytokines: IFN- γ , IL-17A, TNF- α , IL-2 and IL-10. No differences in the frequency of total $CD3^+$, $CD4^+$ or $CD8^+$ T cells producing IFN- γ , TNF- α , IL-2 or IL-10 were observed between uninvolved intestinal tissue and tumour tissue (Figure 2A-C). There was a significant increase in the frequency of total $CD3^+$ ($p<0.01$) and $CD4^+$ ($p<0.05$) T cells producing IL-17 (Figure 2A-B), indicating that the local microenvironment of colorectal tumours can selectively enhance Th17 cell polarisation or recruitment.

3.3 Tumour infiltrating Th17 cells co-produce TNF- α but not IFN- γ

Having found significantly elevated frequencies of Th17 cells within the pool of lymphocytes recruited to colorectal tumours, we wished to further characterise this T cell subset. Th17 cells have been observed to exhibit plasticity of function and often coproduce IFN- γ , particularly at sites of inflammation. Cells that produce both IL-17 and IFN- γ are thought to

be more pathogenic in inflammatory disease, but conversely would be more effective anti-tumour effectors. Therefore we investigated if the tumour infiltrating Th17 cells also produced other cytokines, notably IFN- γ or TNF- α as this would provide crucial information regarding their potential function.

Representative dot plots shown in Figure 3 were gated on tumour infiltrating CD4⁺ T cells and show IL-17 versus IFN- γ (Figure 3A), IL-17 versus TNF- α (Figure 3B), IL-17 versus IL-10 (Figure 3C) and TNF versus IFN- γ (Figure 3D) staining. There was no significant increase in the frequency of CD4⁺ T cells that co-produced IL-17 and IFN- γ (Figure 3E) or TNF and IFN- γ (Figure 3F). In addition there was virtually no co-expression of IL-17 and IL-10 observed in tumour samples (Figure 3C). However, a significant increase in the frequency of IL-17⁺TNF- α ⁺ CD4⁺ T cells was observed in tumour tissue relative to adjacent uninvolved intestine ($p < 0.05$) (Figure 3G). These data suggest that IL-17⁺TNF- α ⁺CD4⁺ T cells may play a role in shaping the local immune microenvironment of colorectal tumours.

Consistent with the above, a significant positive correlation between the frequencies of CD3⁺IL-17⁺ T cells and CD3⁺TNF- α ⁺ T cells was observed ($p < 0.05$) (Table 2). In addition, a correlation between expression of IL-17 and IL-10 within CD3⁺ T cells was found ($p < 0.01$) (Table 2), however this did not appear due to co-expression of these cytokines. However, IFN- γ production by T cells correlated with the production of both TNF- α ($p < 0.01$) and IL-2 ($p < 0.01$) (Table 2). These data suggest that there are few IFN- γ /IL-17 secreting T cells within colorectal tumours; with the majority of Th17 cells co-producing TNF- α . Furthermore, IFN- γ secreting T cells, when present, also secrete IL-2 in addition to TNF- α . Further understanding of the factors that contribute to these alternative cytokine profiles within the tumour infiltrating T cell pool is required.

3.4 Increased expression of PD-1 on T cells within colorectal tumours

Cytokine production is tightly regulated within human tissues by many mechanisms including cell-cell contact via the immune checkpoint, PD-1. Therefore, we investigated the expression of PD-1 on both CD4⁺ and CD8⁺ T cells in adjacent normal tissue and colorectal tumours by flow cytometry. Representative dot plots show staining controls in peripheral blood (Figure 4A-B), and PD-1 expression by colorectal tumour infiltrating CD4⁺ T cells (Figure 4B) or CD8⁺ T cells (Figure 4C). There was a significantly increased expression of PD-1 on both CD4⁺ ($p < 0.01$) (Figure 4D) and CD8⁺ ($p < 0.05$) (Figure 4E) T cells within colorectal tumour tissue relative to adjacent uninvolved tissue. Interestingly, we found that there was a negative correlation between the expression of PD-1 and IFN- γ for both CD4⁺ and CD8⁺ T cells ($p < 0.01$) (Table 3). The expression of PD-1 on total CD3⁺ T cells also correlated negatively with CD4⁺IL-2⁺ ($p < 0.05$), CD8⁺IL-2⁺ ($p < 0.01$) and CD4⁺IFN- γ ⁺TNF- α ⁺ ($p < 0.01$) and CD8⁺IFN- γ ⁺TNF- α ⁺ ($p < 0.05$) T cells (Table 3). In contrast there was no correlation between the expression of PD-1 and IL-17, TNF- α or IL-10 production (Table 3). These data suggest that the increased expression of PD-1 by colorectal tumour infiltrating lymphocytes may have a suppressive effect on both CD4⁺ and CD8⁺ effector T cells secreting IFN- γ , IL-2 or TNF- α but not IL-17 within colorectal tumours.

3.5 Increased frequency of CD4⁺CD25⁺CD127^{lo} and CD4⁺CD39⁺CD25⁺CD127^{lo}FOXP3⁺ Treg cells in colorectal tumour tissue

Treg cells suppress effector T cells and are thought to contribute to the immunosuppressive microenvironment of tumours. Single cell suspensions obtained from both colorectal tumours and adjacent uninvolved tissue were stained for markers of Treg cells, including CD4, CD25, CD127, FOXP3 and CD39 and were analysed by flow cytometry. The gating strategy for the

identification of Treg cell populations is shown in Figure 5A. Lymphocytes were gated on the basis of CD45⁺ and forward scatter versus side scatter, and then CD4⁺ T cells were gated. A population of CD4⁺CD25⁺CD127^{lo} Treg cells were identified, and then these were further gated on their expression of FOXP3 to identify CD4⁺CD25⁺CD127^{lo}FOXP3⁺ Treg cells. The subset of Treg cells expressing CD39 was then identified (Figure 5A). An increased frequency of CD4⁺CD25⁺CD127^{lo} Treg cells was identified in tumour tissue relative to adjacent colon (p<0.05) (Figure 5B), while there was no significant difference in the frequency of CD4⁺CD25⁺CD127^{lo}FOXP3⁺ Treg cells (Figure 5C). However, the frequency of CD39⁺ Treg cells was significantly increased in tumour tissue relative to uninvolved tissue (p<0.05) (Figure 5D). A positive correlation between KM score and the percentage CD4⁺CD25⁺CD127^{lo} Treg cells was observed in tumours (Table 4), suggesting that increased immune infiltration is associated with an increased frequency of Treg cells within the infiltrate. There was a negative correlation between the frequency of CD4⁺CD25⁺CD127^{lo} Treg cells and the expression of IFN- γ and IL-2 in tumours (p<0.05) (Table 4), suggesting that Treg cells may exert a suppressive effect on effector T cell cytokines.

Discussion

In this study we investigated the phenotype of tumour infiltrating T cells by flow cytometry in a cohort of patients with MSS colorectal cancer whose tumours had broadly similar immune cell infiltrate as determined by pathological analysis (KM score). We found increased frequencies of Treg cell populations in the colorectal tumour tissue, which positively correlated with the KM score. It has been established that increased immune infiltration is a positive prognostic factor in colorectal tumours. Our data showing a positive correlation between Treg infiltration and KM score therefore suggests that Treg cell infiltration may also correlate with improved outcomes. Given the prospective nature of our study however clinical outcomes were not yet available for our study cohort. Our findings are in broad agreement with others which showed that increased Treg cell infiltration into colorectal tumours correlated with improved clinical outcomes (14, 15). This is in contrast to the case for the majority of other cancers where Treg cell infiltration tends to be a negative prognostic indicator. Our findings and those of others showing Treg infiltration to be a positive prognostic factor seems counterintuitive since it is generally assumed that Treg cells exert a pro-tumour role by suppressing anti-tumour immune responses. Treg cells have been shown to be enriched in parallel with effector T cells at inflamed peripheral sites within the body (25), most likely as an inherent mechanism to maintain homeostasis. However, for various reasons Treg cells still fail to constrain inflammation at these sites of autoimmune inflammation (25). Colorectal tumours are known to be highly inflammatory relative to other tumour types (26). Thus the enrichment of Treg cells within colorectal tumours may just be a reflection of the increased immune infiltration observed in colorectal tumours rather than an indication of a specific anti-tumour role for Treg cells.

Other studies did not find a positive correlation between good prognosis and Treg cell infiltration (16, 17). The reasons for these conflicting results are not clear. However they may reflect some of the difficulties in identifying Treg cells by immunohistochemistry, since the use of sole Treg markers such as CD25 or FoxP3 can lead to the false identification of activated effector T cells as Treg cells. In our study, using a flow cytometric approach we were able to unequivocally identify Treg cells using a full panel of well accepted Treg markers. It is a matter of debate whether tumour infiltrating Treg cells act to inhibit anti-tumour effector T cells or other inflammatory responses which may be pro-angiogenic and thus pro-tumourigenic (27).

We found that Tregs which expressed the ectonucleotidase CD39 were enriched in colorectal tissue. CD39 which is normally expressed on a subset of human Treg cells in addition to other cells, acts to hydrolyse extracellular ATP to AMP which is then further broken down to adenosine by CD73 (28). Adenosine binds to its cellular receptors, including the A2A receptor on T cells where it exerts a suppressive effect. Thus extracellular ATP, which is elevated in tumours, can be rapidly hydrolysed by CD39 expressing Treg cells to mediate suppressive effects on T cells. It is likely therefore that the CD39⁺ Tregs which we identified in colorectal tumours will have a suppressive effect via adenosine. We also observed an increase in CD25⁺CD127^{lo} Treg cells, which were not all FoxP3⁺, although FoxP3⁺ Treg cells were included in this population. This was in agreement with a study that showed increased frequencies of FoxP3⁺ Treg cells in colorectal tumours which were even more suppressive than FoxP3⁺ Tregs and produced IL-10 and TGF- β (29). The function of Treg cells within colorectal tumours is of key importance, particularly if they are to be considered as targets for immunotherapy. We therefore sought possible correlations between the frequencies of tumour infiltrating Tregs and T cell cytokines. The frequencies of CD25⁺CD127^{lo} Treg cells correlated negatively with total T cell IFN- γ and IL-2, suggesting that these Treg cells may

inhibit anti-tumour effector responses, however, there was no correlation between Treg cells and T cell IL-17 or TNF- α , both of which have been suggested to have pro-tumour activity (30, 31). Thus it is possible that Treg cells are increased in colorectal tumours in proportion to the inflammatory infiltrate, where they may exert differentially suppressive effects on different subsets of T cells. The data suggest that Treg cells may be more effective at inhibiting T cells producing IFN- γ , such as Th1 and CTL than those producing IL-17. Together with other immunosuppressive mechanisms within the tumour, this may help to tip the balance in favour of tumour progression.

Recent reports have described an aberrant subset of ROR γ t⁺/FoxP3⁺ IL-17-producing Treg cells (32). We therefore investigated whether the increased production of IL-17A by CD4 T cells within tumours was derived from FoxP3⁺ Treg cells. We saw that, although the majority of IL-17A in tumours came from FoxP3⁺ CD4 T cells, we also identified a population of IL-17⁺FoxP3⁺ CD4 T cells within tumours (data not shown). We also observed a positive correlation between IL-17 and IL-10 expression, although there was very little co-expression of these cytokines. The reason for the correlation between IL-17 and IL-10 expression is not clear, however it has recently been shown that Th17 cells converted into IL-17⁺IL-10⁺ cells via a TGF- β dependant mechanism (33). This may be a mechanism to downregulate inflammatory Th17 cells in the dynamic tumour environment.

In addition to Treg cells, the expression of PD-1 in tumours also provides important inhibitory signals in the tumour environment, where PD-1 expressing T cells can be rendered anergic by engaging their ligand PDL-1 which is expressed on tumour cells (19). We observed increased expression of PD-1 on both CD4 and CD8 T cells infiltrating colorectal tumours. PD-1 expression correlated negatively with T cell effector cytokines IFN- γ and IL-2, but not with IL-17. Increased expression of PD-1 on tumour infiltrating CD8 T cells correlated with an exhausted phenotype and reduced expression of IFN- γ and IL-2 (34). Thus it appears that both Treg cells and PD-1 expressing cells may serve to inhibit effector T cells that produce IFN- γ and IL-2 but not IL-17, TNF- α and IL-10. Since trials to test the efficacy of immune checkpoint inhibitors such as anti-PD-1 in colorectal cancer are ongoing, it will be crucial to understand the role of PD-1 in colorectal tumours. There appear to be some discrepancies in the literature regarding the levels of PD-1 expressed by tumour infiltrating lymphocytes which are likely due to technical differences between studies. A study reporting that colorectal tumour infiltrating CD8⁺ T cells expressed little or no PD-1 employed a long (12 hour) tissue digestion protocol (35) and this may have impacted on the phenotype of the recovered cells as determined by flow cytometry (36). In contrast, another study which used a shorter digestion period reported similar levels of PD-1 expression to those observed in our study (34). A further study investigating the expression of immune check-point inhibitors in MSI versus MSS colorectal cancer demonstrated PD-1 expression on both CD4 and CD8 T cells (37). Although the levels of PD-1 in the latter study appear lower than those that we observed, the PD-1 data was expressed relative to that of T cells infiltrating normal adjacent colon tissue (PD-1 high). Thus the absolute expression levels of PD-1 on tumour infiltrating T cells appear very similar to those that we observed (37). These discrepancies highlight the need for harmonisation of methods and validation of PD-1 expression levels among different patient cohorts.

IFN- γ produced by both CD8 and CD4 T cells is considered to be a key anti-tumour effector. However we did not observe any global differences in the expression of IFN- γ by CD4 or CD8 T cells in tumour versus adjacent uninvolved tissue. IL-17A which was increased in tumour tissue was the only cytokine with altered expression when compared with adjacent

uninvolved tissue. IL-17A was produced primarily by CD4 T cells which largely co-produced TNF- α . In contrast, only a small proportion of IL-17⁺ cells co-expressed IFN- γ . Th17 cells are known to be plastic and polyfunctional in terms of cytokine production and in the context of autoimmunity coproduction of IL-17 and IFN- γ is thought to be inflammatory and pathogenic, while in tumours such cells might be good anti-tumour effectors. However, the role of Th17 cells in cancer is still controversial, with both pro- and anti-tumour roles having been ascribed to IL-17. In colorectal cancer the consensus appears to be that the expression of IL-17 may be a negative prognostic factor (4). Although IL-17 is an inflammatory cytokine that might be expected to have an anti-tumour effect, it is possible that its inflammatory effects rather serve to promote angiogenesis and promote tumour growth. Indeed, the angiogenic effects of IL-17 via the induction of VEGF have been documented (9). TNF- α also has angiogenic effects (30) thus the Th17 cells that co-produce TNF- α but not IFN- γ , that we have identified as being enriched in colorectal tumour tissue, may be particularly effective at inducing angiogenesis and thereby promoting tumour growth. Our data suggest that these cells may not be constrained by the regulatory networks that are prevalent in the tumour, including CD39⁺ Treg cells and PD-1 expressing T cells. This may have important implications for tumour therapy and a fuller understanding of the role of Th17 cells and their regulation within colorectal tumours is warranted. Furthermore, TNF- α and IL-17A are thought to have synergistic tumour promoting properties when expressed together in colorectal cancer cells, whereby these cytokines stimulate glucose metabolism and growth factor production (38).

Author contributions

MD designed and performed flow cytometric analysis of tumour infiltrating and tissue T cell populations, analysed data and contributed to the drafting of the manuscript and approved the final version.

CR and KDS reviewed the pathology of all cases and performed the KM score analysis. Contributed to drafting of the manuscript and approved the final version.

BN, MT, RG, DCW, PRO'C, JH and GD recruited and consented patients, provided clinical samples, compiled and interpreted clinical information, approved final manuscript.

JF and EJR designed the experiments, drafted the manuscript and approved the final version, and are accountable for the accuracy and integrity of the work.

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538 **Table 1.** Patient Characteristics
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N	22
Male /Female	16 / 6
Age (Years), Median (Range)	69 (47-93)
TMN Stage	Stage I (2), Stage II (9), Stage III (9) Stage IV (2)
Lymphovascular Invasion	Present (11), Absent (11)
Perineural Infiltration	Present (6), Absent (16)
Extramural Venous Invasion	Present (12), Absent (10)
Mismatch Repair Loss	0/22
KM Score	0 (2), 1 (16), 2 (4)
Tumour Budding	Present (10), Absent (12)
Tumour Differentiation	Moderate (17), Well (5)
Tumour Size (cm),Median (Range)	4.8 (1.2-9.5)
Tumour Location	Sigmoid (4), Caecum (4), Ascending (6), Transverse (1), Descending (3), Rectal (4)
Surgery	Curative Intent (21), Hartman's Procedure (1)
Adjuvant Chemotherapy	Yes (10), No (12)
Recommended	

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Table 2. Pearson correlation of IL-17 or IFN- γ production by tumour infiltrating CD3⁺ T lymphocytes versus other cytokines (n=20).

Pearson Correlation vs IL-17⁺ Lymphocytes	
CD3⁺ IFN-γ	0.241
CD3⁺ TNF-α	0.519*
CD3⁺ IL-2	0.164
CD3⁺ IL-10	0.665**
Pearson Correlation vs IFN-γ⁺ Lymphocytes	
CD3⁺ TNF-α	0.602**
CD3⁺ IL-2	0.747**
CD3⁺ IL-10	0.163

*Correlation is significant at the 0.05 level (2-tailed);

** Correlation is significant at the 0.01 level (2-tailed)

547 **Table 3.** Pearson correlation of CD3⁺ T cell cytokine production with tumour infiltrating
548 PD1⁺ T cells (n=11)

CD3 ⁺ Cytokine Producing Cells	Pearson Correlation vs CD4 ⁺ PD1 ⁺ T cells	Pearson Correlation vs CD8 ⁺ PD1 ⁺ T cells
IFN- γ	-0.843**	-0.952**
IL-17	-.402	-0.426
TNF- α	-0.632	-0.492
IL-10	-0.288	-0.149
IL-2	-0.642*	-0.829**
IFN- γ +TNF- α	-0.744**	-0.626*
IFN- γ + IL-17	-0.298	-0.330
TNF- α + IL-17	-0.409	-0.368

549 *Correlation is significant at the 0.05 level (2-tailed);** Correlation is significant at the 0.01 level (2-tailed)

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Table 4. Pearson correlation of CD4⁺CD25⁺CD127⁺ T cells and infiltrating cytokine producing cells in colorectal tumours (n=14)

Pearson Correlation vs CD4 ⁺ CD25 ⁺ CD127 ⁺ T cells	
KM Score	0.518*
IL-17	-0.198
TNF- α	-0.512
IFN- γ	-0.574*
IL-2	-0.598*
IL-10	0.139

*Correlation is significant at the 0.05 level (2-tailed)

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Figure Legends

Figure 1. Colorectal tumours are highly infiltrated with T cells. Representative H&E slides of colorectal tumours with varying KM scores. (A) KM score 0, (B) KM score 1, (C) KM score 2, (D) Crohn's like reaction. Single cell suspensions were also obtained by enzymatic digestion from surgically excised normal adjacent colonic tissue and colorectal tumours. The frequency of CD3⁺ (E), CD4⁺ (CD3⁺CD8⁻) (F) and CD8⁺ (G) T cells within lymphocytes was determined by flow cytometry. *, p<0.05 as determined by the Wilcoxon signed rank test.

Figure 2. Increased frequency of CD4⁺IL-17⁺ (Th17) cells infiltrating colorectal tumour tissue compared to normal adjacent tissue. Tumour tissue and adjacent uninvolved colonic tissue were enzymatically digested and the resulting single cell suspension was stimulated with PMA and ionomycin. Cells were stained with antibodies specific for CD3 and CD8, permeabilised and stained for intracellular expression of cytokines IFN- γ , IL-17, TNF, IL-2 and IL-10. The frequency of cytokine expressing total CD3⁺ (A), CD4⁺ (CD3⁺CD8⁻) (B) or CD8⁺ (C) T cells is shown for uninvolved colon and colorectal tumour tissue. *, p<0.05, ** p<0.01 as determined by Wilcoxon signed rank test.

Figure 3. Th17 cells infiltrating colorectal tumours co-express TNF- α but not IFN- γ . Single cell suspensions that were obtained from tumour tissue and adjacent uninvolved colonic tissue were stimulated with PMA and ionomycin. Cells were stained with antibodies specific for CD3 and CD8, permeabilised and stained for intracellular expression of the cytokines TNF- α , IFN- γ and IL-17. Representative dot plots were gated on CD4⁺ (CD3⁺CD8⁻) T cells and show expression of IL-17 versus IFN- γ (A), IL-17 versus TNF- α (B), IL-17 versus IL-10 (C) or TNF versus IFN- γ (D). The frequencies of CD4⁺IL-17⁺IFN- γ ⁺ (E), CD4⁺TNF⁺IFN- γ (F) or CD4⁺IL-17⁺TNF- α ⁺ (G) T cells are shown for uninvolved colon and colorectal tumour tissue. *, p < 0.05, as determined by the Wilcoxon signed rank test.

Figure 4. Increased frequency of PD-1 expression on tumour infiltrating CD4⁺ and CD8⁺ T cells. Tumour tissue and adjacent uninvolved colonic tissue was digested and stained with antibodies specific for CD3, CD4, CD8 and PD-1. Representative FMO control (A) and PD-1 dot plots of PD-1 staining on CD3⁺ T cells in blood (B), PD-1 staining on CD4⁺ (B) and CD8⁺ (C) T cells in uninvolved colon or tumour samples are shown. The graphs show the frequency of PD-1⁺ CD4⁺ (D) and CD8⁺ (E) T cells. *, p < 0.05, **, p<0.01 as determined by the Wilcoxon signed rank test.

Figure 5. Increased frequency of CD4⁺CD25⁺CD127^{lo} and CD4⁺CD25⁺CD127^{lo} FoxP3⁺CD39⁺ Treg cells in colorectal tumour tissue. Tumour tissue and adjacent uninvolved colonic tissue was enzymatically digested to form a single cell suspension which was stained with antibodies specific for CD45, CD4, CD25, CD127, FOXP3 and CD39 and analysed by flow cytometry to identify Treg cell populations. The sequential gating strategy used to identify Treg cell populations is shown in (A). The graphs show the frequency of CD4⁺CD25⁺CD127^{lo} (B), CD4⁺CD25⁺CD127^{lo}FOXP3⁺ (C) and

596 CD4⁺CD25⁺CD127^{lo}FOXP3⁺CD39⁺ (D) Treg cells. *, p<0.05, as determined by the
597 Wilcoxon signed rank test.

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Figure 1.TIF

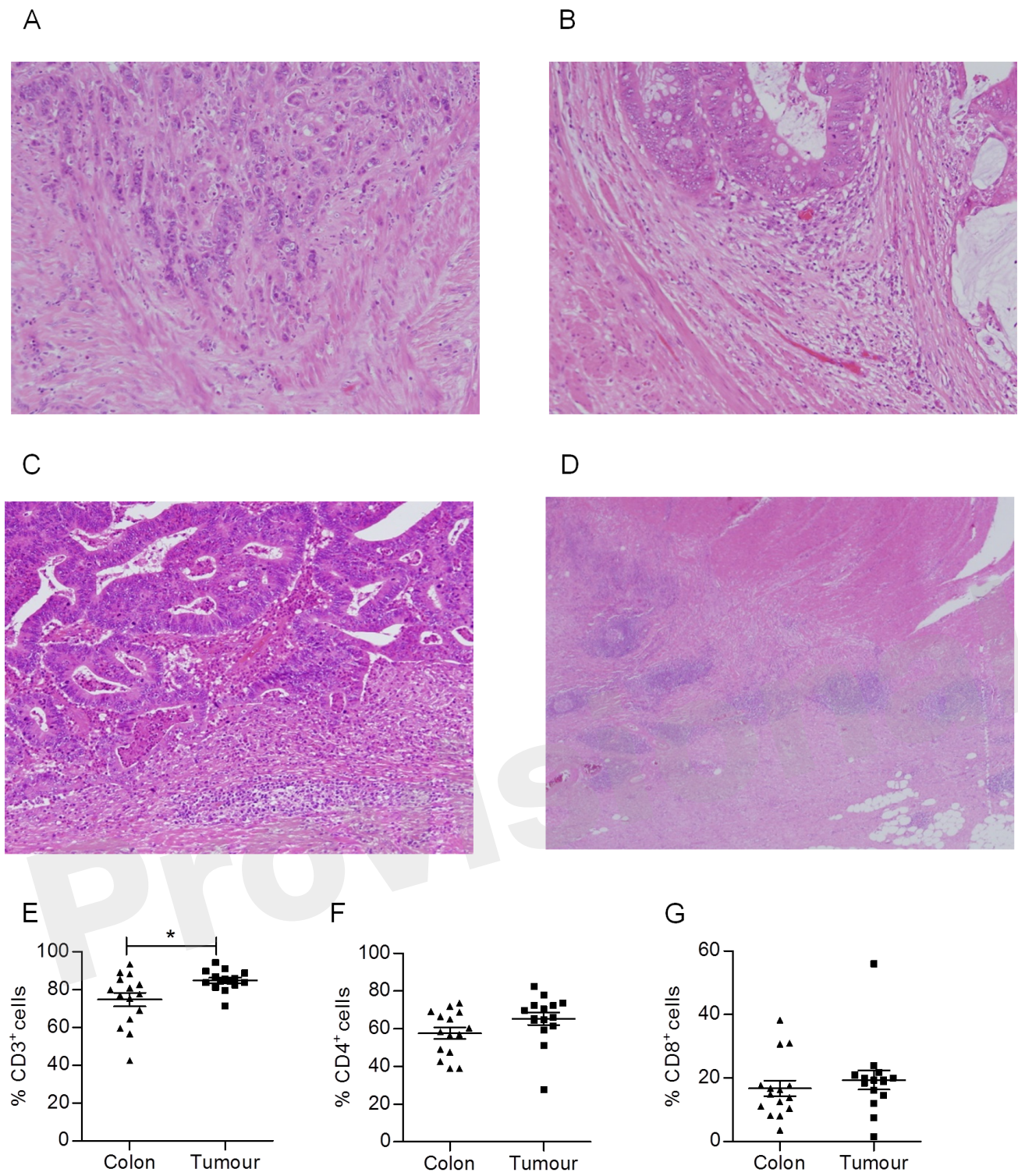


Figure 1

Figure 2.TIF

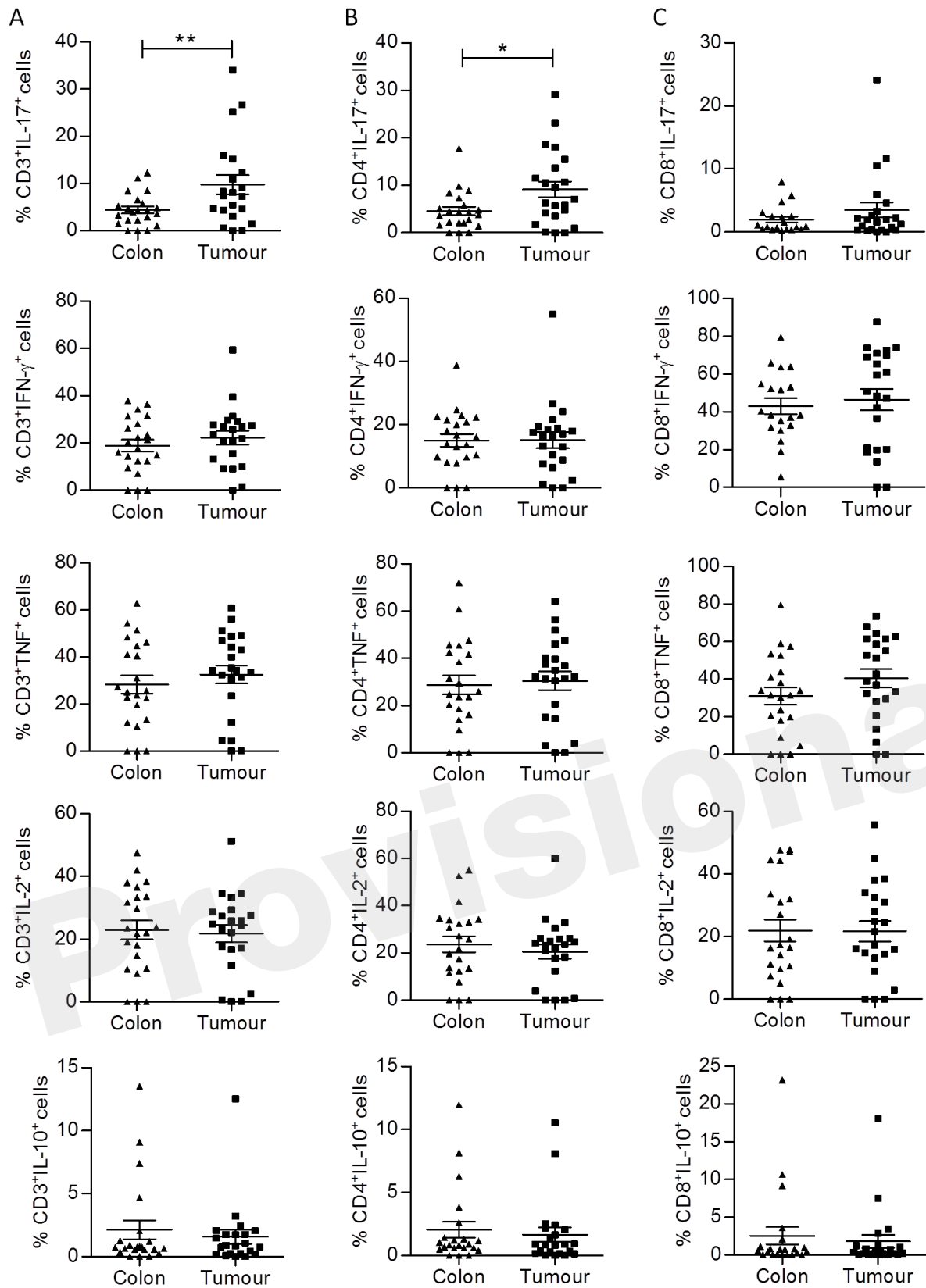


Figure 2

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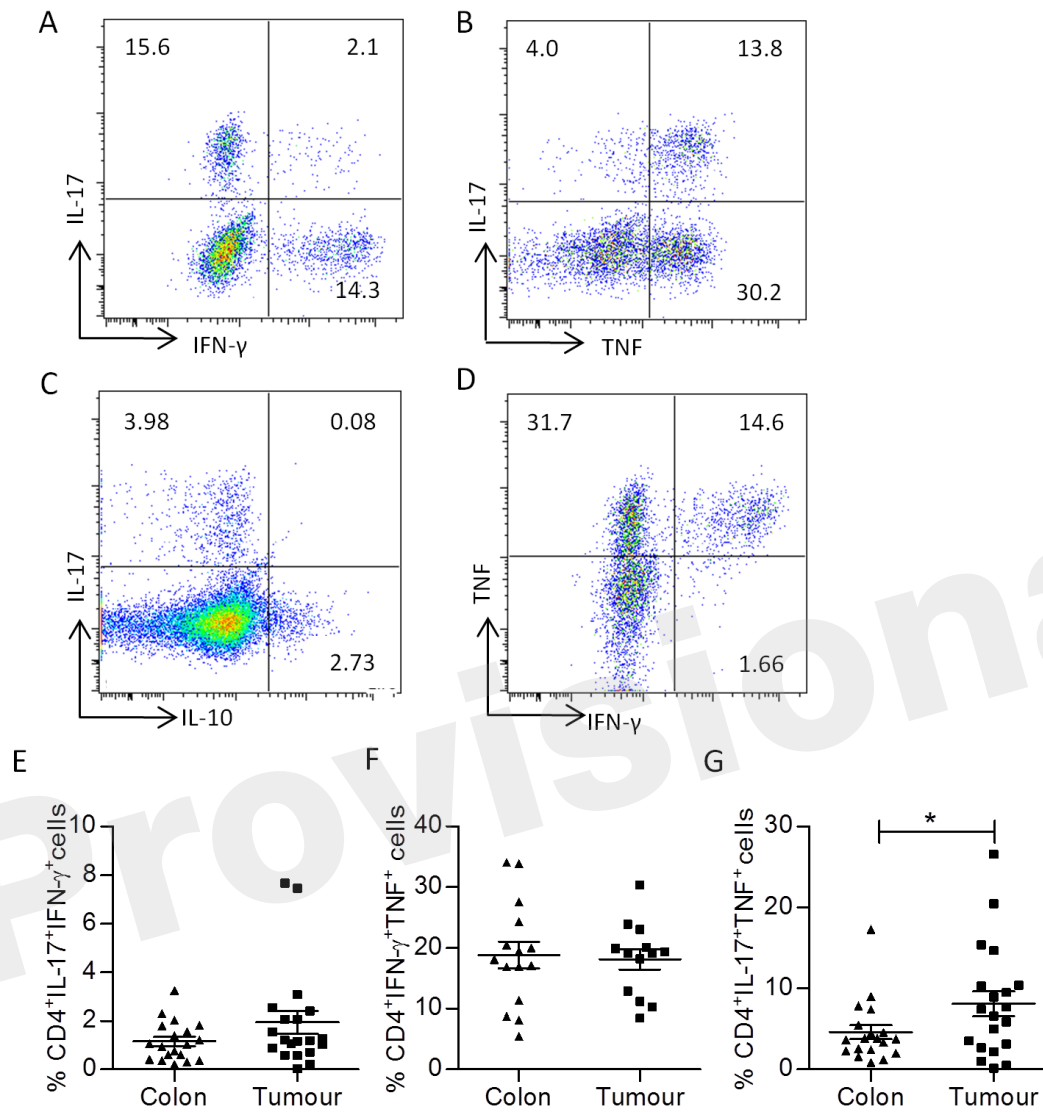


Figure 3

Figure 4.TIF

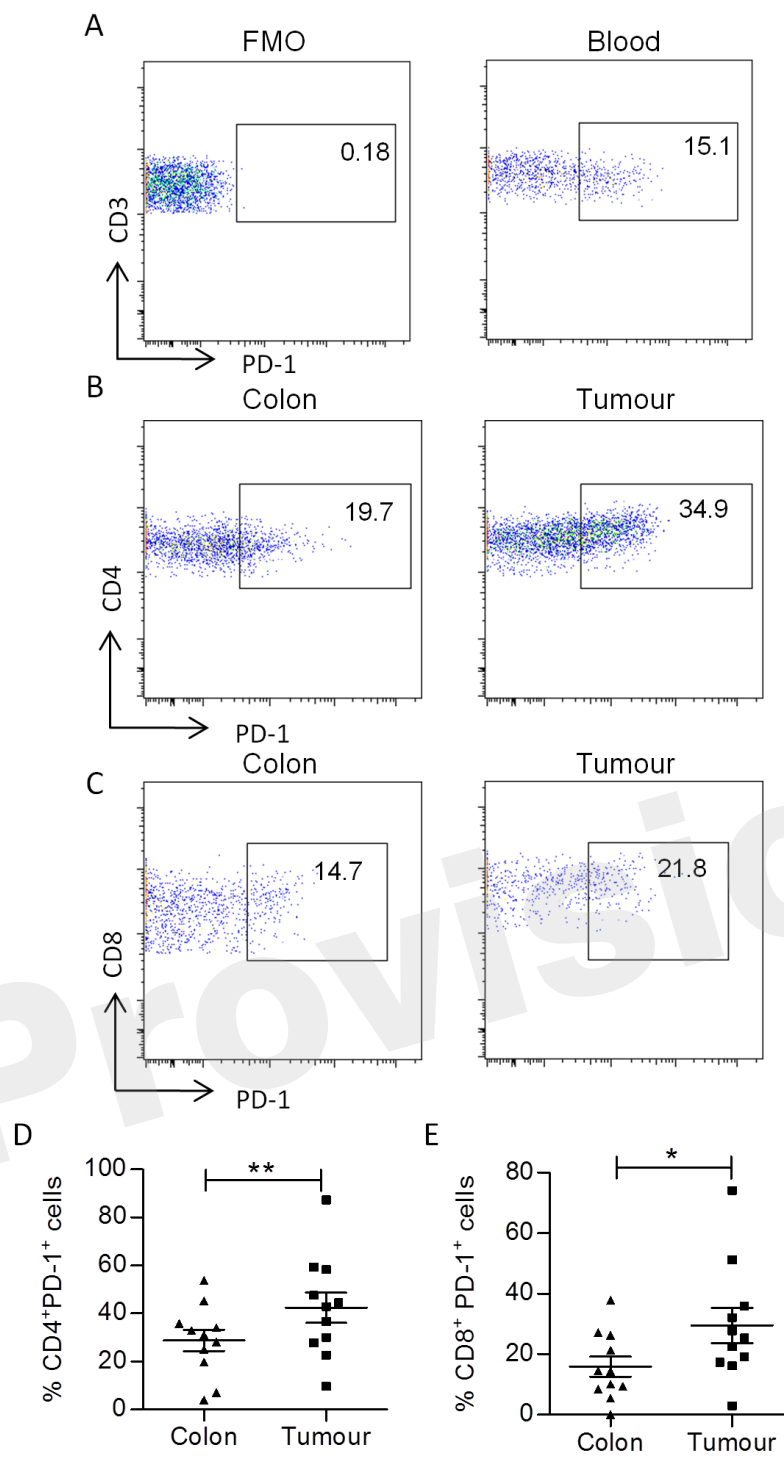
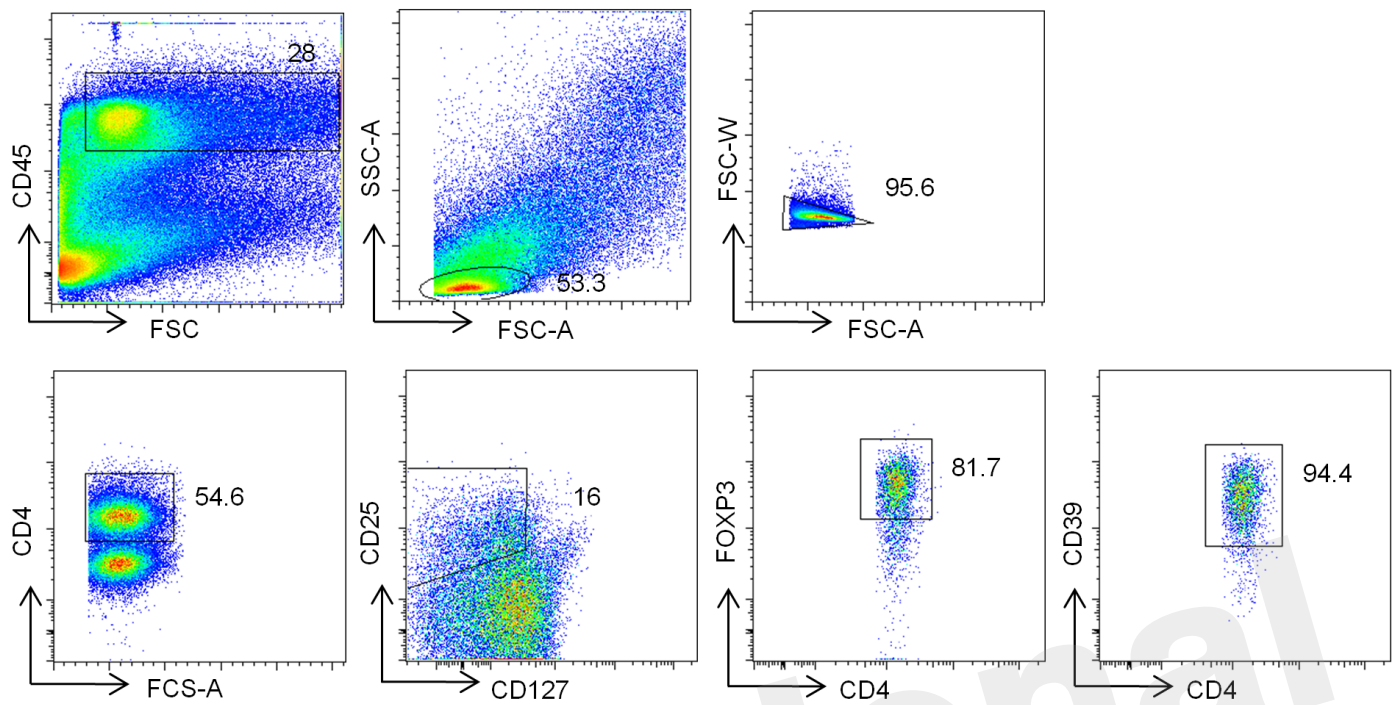


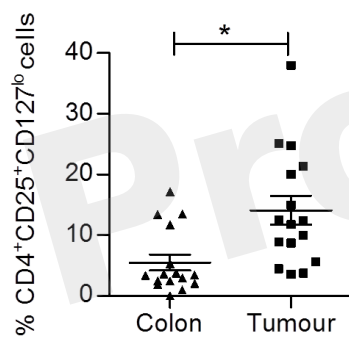
Figure 4

Figure 5.TIF

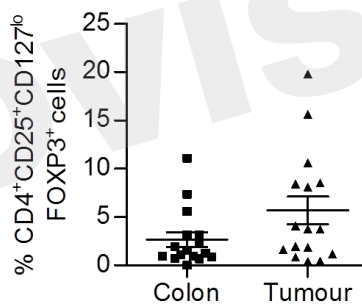
A



B



C



D

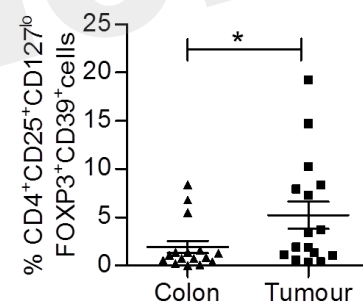


Figure 5