Proximal Signaling Responses in Peripheral T Cells from Colorectal Cancer Patients are Affected by High Concentrations of Circulating Prostaglandin E2

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Short title: T-cell signaling profiles in colorectal cancer patients
Abbreviations: BD; blood donors, CEA; carcinoembryonic antigen, COX; cyclooxygenase, CRC; colorectal cancer, ITAM; immunoreceptor tyrosine based activation motif, MFI; median fluorescence intensity, ND; not determined, NSAID; non-steroidal anti-inflammatory drug, Pat; patients, PBMC; peripheral blood mononuclear cell, PGE$_2$; prostaglandin E$_2$, TCR; T cell receptor.
Abstract

Patients with colorectal cancer (CRC) have been shown to have elevated levels of circulating prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) which promotes cancer progression and suppresses T-cell immune responses. In this study we evaluated whether signaling responses in T lymphocytes obtained from peripheral blood of CRC patients were affected by the sustained exposure to increased levels of PGE\textsubscript{2}. The phosphorylation status of an extended panel of proteins involved in downstream signaling cascades in T cells was profiled at a single cell level both in naïve and antigen-experienced cells after triggering T cell-, prostaglandin- and interleukin-2 receptors. Peripheral T cells from patients with elevated PGE\textsubscript{2} levels displayed aberrant T-cell signaling responses downstream of the T cell receptor (assessed by reduced phospho-CD3\textgreek{z} and SLP76 responses), and after triggering the IL-2 receptor (assessed by reduced phosphorylation of STAT5) when compared to T cells from CRC patients with lower levels of PGE\textsubscript{2} and T cells from healthy blood donors. This signaling study of circulating T cells from CRC patients indicates that increased systemic PGE\textsubscript{2} levels affect proximal T-cell responses and confirm phospho-specific flow cytometry to be a valuable tool for revealing signaling signatures in immunological disorders.

Keywords: Colorectal cancer, PGE\textsubscript{2}, T cell signaling, phospho-specific flow cytometry
1.1 Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in the world [1] and with 42.2 and 36.1 new cases per 100,000 people per year for men and women, respectively, Norway is among the European countries with the highest incidence of CRC [2]. Most cases of CRC are sporadic and are thought to be driven by genetic mutations, epigenetic changes and chronic inflammation, as well as diet and lifestyle behaviors [3]. Colorectal cancer develops partly through adenomas and screening by colonoscopy is an effective, although costly, way to prevent CRC by identifying and removing pre-cancerous adenomas. In line with this, patients diagnosed with localized disease have an 80-90% five-year survival rate while the survival rate drops to under 10% for carcinomas with distant metastasis [4]. Surgery is the standard treatment for CRC that has not spread to distant sites, but patients with advanced and aggressive disease need adjuvant therapies, primarily involving chemotherapy and radiation therapy which may considerably improve prognosis.

The host immune system is capable of mounting immune responses to the tumor cells, mainly through tumor infiltrating T lymphocytes and natural killer cells [5]. However, immune surveillance and activation are frequently ineffective in preventing or controlling tumor growth, and colorectal cancers have been shown to induce immune suppression linked to poorer clinical outcome [6]. Several mechanisms for immune suppression have been described, but the aberrant expression of cyclooxygenase-2 (COX-2) and its role in the production of prostaglandin E$_2$ (PGE$_2$) has a strong association with the development and maintenance of CRC [7]. Cyclooxygenase-2 has been found to be overexpressed in 40% of colorectal adenomas [8] and in up to 90% of carcinomas [9]. Furthermore, we have previously revealed that CRC patients have elevated levels of circulating PGE$_2$ in their blood [10]. The crucial role of COX-2 and PGE$_2$ in CRC carcinogenesis is demonstrated by randomized trials proving regular pharmacological inhibition of the COX-enzymes with non-steroidal anti-inflammatory drugs (NSAIDs) to be effective in reducing both the incidence and progression of intestinal tumors in CRC patients [11]. However, long-term use has been associated with side effects in the gastrointestinal tract and cardiovascular system which has dampened their use as chemopreventive agents. Additional modes of therapy and chemoprevention of CRC that are both effective and tolerable remain to be developed, and the PGE$_2$ signaling pathway is a potential target which necessitates insight into the immunomodulatory role of PGE$_2$ in CRC pathology.
Cyclic adenosine monophosphate (cAMP) is the main intracellular second messenger of PGE\textsubscript{2} and increased production of cAMP is known to inhibit TCR signaling through the protein kinase A (PKA) type I-CSK (COOH-terminal Src kinase)-LCK (lymphocyte-specific protein tyrosine kinase) signaling cascade [12]. The TCR complex lacks intrinsic enzymatic activity, but upon triggering of T cells, the tyrosine kinase LCK rapidly becomes activated and phosphorylates tyrosine residues within specialized motifs (immunoreceptor tyrosine based activation motifs; ITAMs) in the cytoplasmic tail of the signal transduction unit of the TCR/CD3 complex, the CD3 chains. Upon T-cell activation, PKA type I redistributes to and co-localizes with the TCR/CD3 complex [13] and phosphorylates its TCR proximal substrate CSK at serine 364, resulting in increased CSK activity [12]. CSK subsequently provides negative control of LCK by phosphorylating the inhibitory tyrosine 505 [14]. As a result, downstream T-cell signaling starting with ITAM phosphorylation of the CD3 chains is hindered. Prostaglandin E\textsubscript{2} exerts immune suppression on T cells through multiple modes of action and has also been shown to inhibit the production of the pivotal T cell cytokine interleukin (IL)-2 [15], expression of the IL-2 receptor [16, 17] and IL-2 signal transduction elements [18] resulting in diminished IL-2 stimulated proliferation responses.

The present study was designed to find out whether the levels of circulating PGE\textsubscript{2} from the tumor and immune system in patients with CRC affect stimulation responses of peripheral T cells. Blood was drawn from patients not undergoing adjuvant therapy or receiving medication inflicting with prostaglandin synthesis and from healthy blood donors. We measured PGE\textsubscript{2} levels and profiled signaling cascades in various T cell subsets after triggering T-cell-, prostaglandin- and interleukin-2 receptors in a phospho-specific flow cytometric setup.

1.2 Materials and Methods

Patient material

The study was approved by the Regional Committee for Medical Research Ethics, for SouthEastern Norway and conducted in compliance with the Declaration of Helsinki. Patients with colorectal cancer at the Department of Gastrointestinal Surgery, Oslo University Hospital, were enrolled in the study after obtaining written informed consent. 11 patients were enrolled in the study and ten of the patients were included in the final analysis (six women and four men; average age 67.2 years; range 37-90). Patients included were not medicated with steroids (inhibitors of arachidonic acid and prostaglandin synthesis), NSAIDs
(inhibitors of COX-enzymes) and had not previously received chemo- or radiation therapy. Levels of carcinoembryonic antigen (CEA) were measured for six of the patients by ELISA (Roche, Basel, Switzerland) at the Department of Medical Biochemistry, Oslo University Hospital. Blood samples from five healthy blood donors (Blood Bank, Oslo University Hospital) were used as controls.

1.2.1 Isolation and stimulation of cells
Peripheral blood (approximately 50 ml) was drawn from patients pre-operatively. Peripheral blood mononuclear cells (PBMCs) (24-100×10^6 cells) from patients and blood donors were isolated by Isopaque-Ficoll (Lymphoprep, Axis-Shield PoC, Oslo, Norway) gradient centrifugation. Plasma was isolated from the samples and frozen at -80 °C in order for PGE_2 levels to be measured at the same time for all study objects. Cells were re-suspended in RPMI 1640 medium (Life Technologies, Carlsbad, CA, USA) supplemented with 1% fetal bovine serum, 1 mM sodium pyruvate, 0.9% (v/v) MEM non-essential amino acids, 100 U/ml penicillin and 100 U/ml streptomycin for cell stimulation (all Life Technologies). Cells were subsequently left unstimulated or subjected to five different stimulation conditions at 37 °C (see Fig. 2A); (1) PGE_2 (10 µM, Sigma-Aldrich, St. Louis, MO, USA) for 10 min, (2) cross-linking of biotinylated anti-CD3 (1 µg/ml, clone OKT-3, custom affinity-purified and biotinylated by Diatec Monoclonals AS, Oslo, Norway) and biotinylated anti-CD28 (5 µg/ml, clone CD28.2, eBioscience, San Diego, CA, USA) with avidin (50 µg/ml, Zymed Laboratories, Life Technologies) for 1 min, (3) a combination of PGE_2 (10 µM, 10 min) and TCR co-stimulation (anti-CD3/CD28, 1 min), (4) IL-2 (100 IE, Sigma-Aldrich) for 30 min or (5) a combination of PGE_2 (10 µM, 40 min) and IL-2 (100 IE, 30 min).

1.2.2 Prostaglandin E_2 determination
Peripheral blood from CRC patients as well as control samples were collected in EDTA vacuum tubes and centrifuged. Plasma was subsequently isolated and stored for 3-18 months at -80 °C, thawed and subsequently analyzed for PGE_2 levels by ELISA (R&D, Minneapolis, MN, USA) according to manufacturer’s description. All samples were analyzed simultaneously in duplets or triplicates.
1.2.3 Phospho-specific flow cytometric analysis

Stimulation conditions were titrated as described in Kalland et al., 2011 [19] for anti-CD3/CD28 co-stimulation and as described in Oberprieler et al., 2010 [20] for PGE$_2$ treatment. After stimulation, cells were fixed immediately using pre-warmed formaldehyde (BD Phosflow Fix Buffer I, BD Biosciences, USA) for 10 min at 37 °C, permeabilized in methanol (BD Phosflow Perm Buffer III, BD Biosciences, San Jose, CA, USA) and stored at -80 °C. Cells from one patient and one control were then thawed and subjected to two-dimensional fluorescence cell barcoding (FCB) with Pacific Blue and Alexa Fluor 488 or Pacific Orange (all Molecular Probes, Life Technologies), providing each sample with a unique fluorescent signature as described in Krutzik et al., 2006 [21]. Cells were washed and combined, and the pooled samples were stained with phospho-specific and surface marker antibodies for 30 min at room temperature. PerCP conjugated CD3, PE-Cy7 conjugated CD4 and PE conjugated CD45RO were all from BD Biosciences. All antibodies used to detect the phosphorylation status of the proteins were conjugated with Alexa Fluor 647. Antibodies used to detect the phosphorylation of CD3ζ (Y142), LAT (Y171), MEK1 (S298), NF-κB p65(S529), SAPK/JNK (T183/Y185), SLP76 (Y128), STAT1 (Y701), Rb (S807/S811), STAT1 (S727), STAT3 (Y705), STAT5 (Y694), ZAP70/Syk (Y319/Y352) and the isotype control IgG1κ were from BD Biosciences. Antibodies used to detect the phosphorylation of 44/42 MAPK (T202/Y204), AKT (S473), AKT (T308), AKT substrate (RXRXXS/T), GSK3α (S21), GSK3β (S9), histone H3 (S10), HSP27 (S78), MAPK substrate (PXTP), MAPKAPK-2 (T334), NF-κB p65 (S536), p38 MAPK (T180/Y182), PKA substrate (RRXS/T), S6-ribosomal protein (S235/S236), tyrosine (Y100), VASP (S157), VASP (S239) were from Cell Signaling Technology (Danvers, MA, USA). The antibody used to detect the phosphorylation of VAV1 (Y174) was from Santa Cruz Biotechnology (Dallas, TX, USA). Cells were washed and subsequently analyzed by flow cytometry with a FACSCanto II flow cytometer (BD Biosciences) equipped with 407 nm, 488 nm and 633 nm lasers. For fluorochrome compensation, PerCP conjugated CD3 antibody, PE-Cy7 conjugated CD4 antibody, PE-conjugated CD45RO antibody and Alexa Fluor 647 conjugated CD3ζ antibody were added to compensation beads (CompBeads Compensation Particle Set, BD Biosciences), whereas aliquots of unstimulated cells were labeled with the highest concentrations of all fluorescent dyes as compensation controls for the fluorescent dyes. Unstimulated, non-FCB cells were used as reference for unstained cells. Compensation corrections for spectral overlap were automatically set using FACSDiva software (BD Biosciences). For each sample,
at least 20,000 events from each cell population were recorded. Data were analyzed using the Cytobank program (https://www.cytobank.org/) and signaling responses were calculated as the fold change in median fluorescence intensity of treated versus unstimulated cells displayed on the arcsinh transformed ratio [see 21].

1.2.4 Statistical analysis
Data were analyzed using SigmaPlot 11.0 (Systat Software Inc., San Jose, CA, USA). Median values were compared with Mann-Whitney Rank Sum test and with Student T-test for pair-wise comparisons when normally distributed (Fig 3B). p < 0.05 was considered significant.

1.3 Results
1.3.1 Patient characteristics
Eleven CRC patients were enrolled in the study, but one patient was excluded when the histopathological diagnosis was B cell lymphoma of the gut rather than CRC. Clinical characteristics of patients are presented in Table 1. TNM staging (T; tumor invasion, N; lymphatic node, M; metastasis, combined with numbers from 0 to 4 indicating increasing severity) according to the American Joint Committee on Cancer showed that nine out of ten patients were staged T3 or T4, meaning that the tumor had grown into the outermost layers of the colon or rectum or into organ structures. Half of the patients had spreading to regional lymph nodes whereas only one patient had distant metastasis. No correlation between PGE2 levels and number of positive lymph nodes or with levels of the tumor marker carcinoembryonic antigen (CEA) could be observed.

1.3.2 Plasma concentrations of PGE2 and phenotypic characterization of T cell subsets
Examining plasma concentrations of PGE2 it was clear that the patient group was not homogenous, but could be stratified into two groups, one with significantly higher plasma concentrations of PGE2 (n=6; > 1000 pg/ml) than the other (n=4; < 1000 pg/ml) (Fig. 1A). T-cell signaling was therefore separately analyzed for each of these groups. Furthermore, PGE2 levels in the patient group with low levels of PGE2 were not significantly different from those of healthy blood donors that were included as controls (Fig. 1A).
Activation of T lymphocytes occurs in lymphoid tissue and generates functionally diverse subsets of effector T cells that can be phenotypically distinguished by the expression of CD45RO. All patient and control samples were therefore subjected to flow cytometric analysis of their surface expression of CD4 and CD45RO for a more detailed subset analysis of CD4 and CD8 (identified as CD4\(^-\)) and CD45RO\(^-\) naïve T cells and CD45RO\(^+\) effector/memory T cells (Fig. 1B). However, stratification on PGE\(_2\) levels did not reveal any differences in distribution of specific T cell subsets (Fig. 1C).

### 1.3.3 Signal network mapping in CRC patient samples

Earlier studies from our group have mapped PGE\(_2\) pathways in lymphoid cells from healthy individuals [20], which established a framework for analysis of PGE\(_2\) signaling in diseased cells. We therefore set out to map peripheral T-cell signaling responses in patients with CRC known to have elevated plasma levels of PGE\(_2\). Stimuli triggering T-cell responses; TCR co-stimulation, IL-2 or PGE\(_2\), in combination with surface markers and 30 phospho-epitope specific antibodies enabled a thorough phospho-specific flow cytometric analysis of downstream T-cell signaling cascades (Fig. 2A). A selection of the readouts for a representative CRC patient classified with low levels of circulating PGE\(_2\) are displayed in the heatmaps in Fig. 2B and 2C. Square color intensity represents fold change in median phosphorylation of stimulated cells relative to the level in unstimulated cells (arcsinh scale, a delta of 1.75 corresponds to a 10-fold change). In parallel, the same protocol was applied to blood samples from healthy blood donors. Not all monitored phosphorylation parameters responded to the various treatments. Consequently, non-informative readouts were excluded from the rest of the analysis. T-cell signaling analysis from the CRC patient group as a whole versus blood donors did not reveal any significant signaling differences (data not shown). We therefore next stratified the group on PGE\(_2\) plasma concentrations, as accounted for in the previous section, into separate groups of samples from patients with low and high levels of PGE\(_2\). This enabled identification of PGE\(_2\) sensitive signaling nodes in T lymphocytes from CRC patients.

### 1.3.4 Plasma levels of PGE\(_2\) affect proximal T cell receptor signaling events

We found phosphorylation of CD3ζ after one minute of TCR/CD28 co-stimulation to be significantly reduced in the CRC patient samples with elevated plasma levels of PGE\(_2\) when compared to CRC patient samples with lower levels of PGE\(_2\) and to samples obtained from
healthy blood donors (Fig. 3A). The same tendency was observed in all T cell subsets investigated, although not significant for all comparisons. To test whether the cells were exhausted with respect to PGE$_2$ responsiveness by endogenous stimuli, we also pre-incubated the cells with 10 µM exogenous PGE$_2$ prior to one minute of anti-CD3/CD28 co-stimulation. The phosphorylation of CD3ζ was then compared pairwise to responses in the samples from the same individual without addition of PGE$_2$ prior to TCR/CD28 co-stimulation. A significant reduction in phosphorylation of CD3ζ was found in all samples (Fig. 3B).

A key signaling event following TCR stimulation is activation of the adaptor molecules LAT (linker for activation of T cells) and SLP76 (SH2 domain containing leukocyte phosphoprotein of 76 kDa). LAT and SLP76 create docking sites for other adaptors and signal relay molecules, thereby linking the TCR to downstream signaling events [23]. Phosphorylation of SLP76 clearly mirrored the results obtained for CD3ζ (Fig. 3C) where CRC patient samples measured to have elevated PGE$_2$ levels revealed reduced responses in SLP76 phosphorylation. Basal phosphorylation of both CD3ζ and SLP76 proteins were negligible in all subsets and for all groups of study participants (data not shown).

1.3.5 The PKA signaling node of CRC patients with elevated plasma levels of PGE$_2$ was not affected

Activation of PKA by cAMP results in phosphorylation of nearby target substrates on serine or threonine residues presented in a preferred amino acid sequence context of arginine-arginine-X-serine/threonine (where X denotes any amino acid) [24]. The anti-phospho PKA substrate antibody recognizes phosphorylated PKA substrates and was used to assess PKA activation in response to PGE$_2$ treatment (Fig. 4A). We have previously reported maximum response for phosphorylation of PKA substrates to occur ten minutes after exposure to 10 µM PGE$_2$ [20] and this stimulation condition was therefore chosen for our study samples. However, we did not observe blunted responses in the PKA signaling node in CRC patient samples with higher PGE$_2$ plasma levels. The levels of phosphorylation of direct targets of PKA such as VASP (vasodilator-stimulated phospho-protein) and GSK (glycogen synthase kinase) 3α (Fig. 4C and 4E, respectively) were neither affected. Variability among donor samples complicated conclusive readouts for the phospho-epitopes in the PKA signaling node.

When comparing basal phosphorylation levels of proteins in the PKA signaling node, the CD4$^+$ CD45RO$^+$ cells generally exhibited higher basal levels of activation than the other subsets (Fig. 4B, 4D and 4F). This observation has previously been reported from our group
and was found to correlate with suppressed proximal TCR signaling [20], thus indicating that the PKA activity might prevent inappropriate activation of effector/memory cells.

Of note, stimulation with PGE$_2$ alone did not directly affect the phosphorylation status of TCR proximal signaling molecules investigated in the present study (see Fig. 2B).

1.3.6 Long-term exposure to PGE$_2$ inhibits IL-2-specific phosphorylation of STAT5

Signal disturbance caused by PGE$_2$ on the IL-2 receptor pathway was tracked by IL-2-induced phosphorylation of the transcription factor STAT5 (signal transducer and activator of transcription 5). T cells isolated from patients with increased levels of PGE$_2$ responded more poorly to IL-2 treatment than T cells from control groups (Fig. 5A). Furthermore, we also analyzed evoked phospho-STAT5 responses after adding PGE$_2$ on top of IL-2 stimulation, but found no apparent changes (data not shown). Another observation was the higher basal level of STAT5 phosphorylation in the CD4$^-$ population (Fig. 5B).

1.4 Discussion

Prostaglandin E$_2$ is one of the most important biologically active prostaglandins found throughout the gastrointestinal tract [26] and promotes cell proliferation, angiogenesis, migration, tumor invasion and inhibition of apoptosis [27]. Consequently, PGE$_2$ also influences on the carcinogenesis and progression of colorectal cancer. Our earlier studies revealed that elevated circulating levels of PGE$_2$ are found in a proportion of patients with both primary and metastatic CRC [10, 28] and that PGE$_2$ levels take months to return to normal after surgery. The short half-life of PGE$_2$ indicates that COX-2 positive tumors are not the only source of PGE$_2$, a significant part of the PGE$_2$ production probably comes from inflammatory cells. Furthermore, we demonstrated that PGE$_2$-mediated suppression of anti-tumor immune responses takes place both in primary and metastatic cancer and that the level of such suppression predicts clinical outcome [10, 28]. With this background, it was of interest to assess whether elevated PGE$_2$ levels correlated with perturbed signaling downstream of the TCR and IL-2 receptor in peripheral T cells from CRC patients. Our present study supports the notion that PGE$_2$ builds up an immunosuppressive network, also affecting the host at systemic levels.

Six out of ten CRC patient samples exhibited elevated PGE$_2$ plasma levels (Fig. 1A). Dysregulated expression of COX-2 is considered the major determinant of PGE$_2$ levels in
CRC, but specific prostaglandin E synthases have like COX-2 been found to be up-regulated in colorectal tumors [29]. Enzymes catalyzing degradation of prostaglandins are less evaluated in relationship to tumor progression, but loss of 15-hydroxyprostaglandin dehydrogenase (15-PGDH) has been reported in colorectal tumors [30]. A role for differential expression of the four PGE2 receptors (EP1-4) during human colorectal tumorigenesis has also been implied. Colorectal cancer cells and their neighbor cells have been shown to have augmented expression of receptors EP2 [31] and EP4 [32] that stimulate cAMP production upon activation, whereas the EP3 receptor expression, known to inhibit cAMP production upon activation, is often lowered in target cells [33]. Collectively, these data reflect that the impact of PGE2 in CRC pathogenesis is caused by an imbalance in PGE2 synthesis and turnover and in the pattern of receptor expression.

The most profound signaling differences in the present study were found in the phosphorylation of CD3ζ after one minute of TCR/CD28 co-stimulation, elevated plasma levels of PGE2 were associated with reduced phosphorylation of CD3ζ (Fig. 3A). Prostaglandin E2 signals mainly in a cAMP-dependent fashion in T cells and inhibits TCR signaling through the PKA type I-CSK-LCK pathway [12, 14]. Since phosphorylation of CD3ζ in all samples were responsive to the addition of exogenous PGE2, independent of the endogenous levels of PGE2, the inhibitory pathway did not seem to be fully desensitized in any population. Immune dysfunction in T cells from CRC patients and tumor-bearing mice have otherwise previously been suggested to be caused by loss of key signaling molecules associated with TCR, such as CD3ζ and LCK [34-36].

Prostaglandin E2 acts as a second messenger related to G-protein coupled receptors and in response to repeated or long-lasting stimuli, second messenger signals are often dampened due to negative feedback loops or desensitization mechanisms [37]. Down-regulation of EP receptors or PGE2 signaling elements after prolonged exposure of PGE2 could therefore be a plausible explanation for the non-conclusive readouts in PGE2 signaling targets (Fig. 4).

Interleukin-2 is a potent T cell mitogenic stimulus and has been exploited as a means to boost immune responses to cancer, but has shown no clinical benefit in CRC [38]. Our study is in agreement with accumulated evidence from several groups indicating that increased PGE2 levels exert negative control on IL-2 stimulated responses, assessed by IL-2-induced activation of STAT5 (Fig. 5A) [15-18, 39]. The IL-2 receptor functions as a heterotrimer and the high affinity IL-2R contains all three subunits, IL-2Ra, β and the common γc. Expression and dimerization of the β and γc chains alone are sufficient to
mediate IL-2 dependent signaling [40]. We found IL-2 to induce higher phosphorylation responses of phospho-STAT5 in the memory compartment. This can be explained by their constitutive expression of both IL2-Rβ and γc, contrary to naïve cells which require activation-induced up-regulation of the β chain [41].

In conclusion, despite a small sample size we found that proximal T-cell phosphorylation cascades were blunted in CRC patient samples with elevated PGE$_2$ levels. Our findings thereby add to previous results on PGE$_2$ as a negative regulator of T-cell signaling and a key mediator of immunopathology in cancer. However, PGE$_2$-regulated networks are likely to act in concert with other important signaling pathways not investigated in this study that become dysregulated in CRC. Furthermore, we confirmed phospho-specific flow cytometry to be a valuable tool for revealing specific signaling signatures of T-cell activation that could reflect regulation of anti-tumor immunity in CRC.

1.5 Author Contributions
Conceived and designed the experiments: NGO BAB KT. Performed the experiments: KM KH NGO. Analyzed the data: KM KT. Contributed reagents/materials/analysis tools: BAB KT. Wrote the paper: KM KT. Critically reviewed the final manuscript: All authors.
1.6 References


Table Legend

Table 1. Patient characteristics. Clinical, histopathological and laboratory data on the ten CRC patients included in the data analysis. CEA; carcinoembryonic antigen, ND; not determined.
Figure Legends

Figure 1. Levels of circulating PGE₂ and phenotypical characterization of T cell subsets in the study population. A) Levels of PGE₂ (pg/ml) were measured in plasma from patients (Pat) with CRC prior to surgery (n=10) and healthy blood donors (BD) (n=5). Freshly isolated plasma was frozen at -80 °C before PGE₂ was analyzed by ELISA. The study population was subsequently stratified on levels of PGE₂ into groups with low (Pat PGE₂ low) and high PGE₂ (Pat PGE₂ high) levels as indicated for each boxplot. p < 0.01, Mann-Whitney Rank Sum test. B) Gating strategy of the T cell subsets. First, a FSC/SSC plot was made and lymphocytes were identified. The lymphocyte gate was copied to a SSC/CD3 plot identifying CD3⁺ lymphocytes. Then, the CD3⁺ lymphocyte gate was copied to a CD4/CD45RO plot that identified CD4⁺ and CD4⁻ naïve (CD45RO⁻) and effector/memory (CD45RO⁺) cells. C) After isolation of peripheral blood mononuclear cells (PBMCs), CD3⁺ lymphocytes from the study participants were phenotypically characterized based on their surface expression of CD4 and CD45RO molecules. Mean ± SEM is shown.

Figure 2. Phospho-specific signaling profiles of peripheral T cells from CRC patients. A) Workflow of the phospho-specific flow cytometric analysis. Blood was drawn from CRC patients before surgery (n=10), PBMCs were isolated and samples were split into various stimulation conditions regulating T cell responses. Following stimulation, cells were fixed with formaldehyde and subjected to two-dimensional barcoding with increasing concentrations of amino-reactive dyes which allowed pooling of the samples. Cells were then permeabilized with methanol for gaining access to intracellular phospho-epitopes of a range of proteins. Signaling responses were measured by flow cytometric analysis. B) Heatmap depicting phospho-specific responses for a selection of the proteins included in the analysis of the CD3⁺ population from a CRC patient with non-elevated (low) levels of PGE₂. Heatmap is colored according to the change in median fluorescence intensity (MFI) of stimulated relative to unstimulated samples on the archs inh scale (annotated as arcsinh median difference). C) Heatmap displaying phospho-specific antibody signals for CD3ζ (pY142) (upper panel) and PKA substrates (RRXpS/pT) (lower panel) in various T cell subsets.

Figure 3. Aberrant proximal T cell receptor signaling in CRC patients with increased levels of circulating PGE₂. A) Phosphorylation of the CD3ζ chain (pY142) after one minute
of CD3/CD28 co-stimulation of T cells from CRC patients with high levels of circulating PGE$_2$ ($n=6$) compared to T cells from patients with low levels of PGE$_2$ ($n=4$), to healthy blood donors ($n=5$) and to the total population with low PGE$_2$ levels ($n=9$). Cells were incubated with biotinylated anti-CD3 (OKT3, 1 µg/ml) and anti-CD28 (CD28.2, 5 µg/ml) before subsequent cross-linking with avidin (50 µg/ml) for one minute. Responses of the stimulated sample were compared to the unstimulated sample within each subpopulation. Mean ± SEM is shown. * p < 0.05, # p < 0.01 (Mann-Whitney Rank Sum test). B) Percentage of reduction in phosphorylation of CD3ζ (pY142) in all T cell subsets when adding 10 µM exogenous PGE$_2$ prior to one minute of CD3/CD28 co-stimulation compared to phosphorylation of CD3ζ (pY142) in A. Mean ± SEM is shown. One minute of anti-CD3/CD28-induced phosphorylation of CD3ζ (pY142) was also compared pairwise to responses in the same samples pre-incubated for ten minutes with 10 µM exogenous PGE$_2$ prior to CD3/CD28 co-stimulation. ★ p < 0.05 (paired t-test). C) Cells were analyzed for SLP76 phosphorylation (pY128) as in A. Mean ± SEM is shown. Pat; patients, BD; blood donors.

Figure 4. Plasma levels of PGE$_2$ in CRC patients do not affect PKA signaling in peripheral T cells. A, C, E) Levels of PKA substrate (RRXpS/pT), VASP (pS157) and GSK3α (pS21) phosphorylation after ten minutes incubation with exogenous PGE$_2$ (10 µM) in T cells from CRC patients with high levels of circulating PGE$_2$ ($n=6$) compared to patients with low levels of PGE$_2$ ($n=4$), to healthy blood donors ($n=5$) and to the total population with low PGE$_2$ levels ($n=9$). Responses of stimulated sample were compared to unstimulated sample within each subpopulation. Mean ± SEM is shown. B, D, F) Basal levels of phosphorylation of PKA substrates (RRXpS/pT), VASP (pS157) and GSKα (pS21) in T cell subsets compared to the CD4$^+$CD45RO$^-$ population. Mean ± SEM is shown. Pat; patients, BD; blood donors.

Figure 5. Abrogated IL-2-induced STAT5 phosphorylation in CRC patient samples with high levels of circulating PGE$_2$. A) STAT5 (pY694) phosphorylation after IL-2-stimulation (100 IE, 30 min) in T cells from CRC patients with high ($n=6$) and low ($n=4$) levels of circulating PGE$_2$, healthy blood donors ($n=5$) and to the total population with low PGE$_2$ levels ($n=9$). Response of stimulated sample was compared to unstimulated sample within each subpopulation. Mean ± SEM is shown. * p < 0.05. B) Basal levels of STAT5
(pY694) in T cell subsets when compared to the CD4\(^+\) CD45RO\(^-\) population. Mean ± SEM is shown. Pat; patients, BD; blood donors.
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Figure 1

A

B

C

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Figure 2

**A**

- Draw blood, isolate PBMC
- Split sample for stimulation
- Fix and barcode
- Combine and perm
- Split sample for antibody staining
- Run flow and analyze data

**B**

- Unstim
- α-CD3/CD28
- PGE₂
- PGE₂+α-CD3/CD28
- IL-2
- PGE₂+IL-2

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**C**

- CD3ζ (pY142)
- CD3+CD4+
- CD3+CD4-
- CD4+CD45RO+
- CD4+CD45RO-
- CD3+CD45RO+
- CD3+CD45RO-
- CD4+CD45RO+
- CD4+CD45RO-

- PKA substr. (RRxP/S/pT)
Figure 5

A. STAT5 (pY694) phosphorylation

- BD
- Pat PGE$_2$ low
- BD+Pat PGE$_2$ low
- Pat PGE$_2$ high

B. Basal levels of STAT5 (pY694) phosphorylation compared to the CD4+CD45RO- population

- BD
- Pat PGE$_2$ low
- BD+Pat PGE$_2$ low
- Pat PGE$_2$ high

Arcsinh median difference

CD4+CD45RO-
CD4+CD45RO+
CD4-CD45RO-
CD4-CD45RO+