T cell metabolic insufficiency explains the dysfunctional immune response in advanced renal cell carcinoma.

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Abstract

Renal cell carcinoma (RCC) is considered an immunogenic tumour with a prominent dysfunctional immune cell infiltrate, unable to control tumour growth. Although tyrosine kinase inhibitors and immunotherapy have improved the outlook for some patients, many individuals are non-responders or relapse despite treatment, and then develop progressive disease, cancer symptoms and will have a reduced life expectancy. Following the limited success of cytokine therapy, immunotherapy using ipilimumab/nivolumab combination or nivolumab single agent checkpoint-inhibitor therapy has been transformational and reiterated the potential for immune manipulation to significantly impact this disease. There is an urgent need to further improve the potential efficacy of these new targeted immunotherapies as not all patients derive benefit. The immune environment created by the tumour recapitulates that present in chronic viral infections in which inappropriate and excessive antigen stimulation leads to intra-tumoral T-cell exhaustion. These exhausted T-cells exhibit defective proliferative capacities and cytokine production and display an overall phenotype of metabolic insufficiency, characterized by extensive mitochondrial alterations. We set out to comprehensively identify mitochondrial defects in exhausted RCC tumour infiltrating lymphocytes (TILs) and correlate these findings with the clinicopathological characteristics of the tumour and the metabolic tumour microenvironment. The emergence of exhausted RCC CD8+ TILs upon progression into p3-4 advanced stage disease coincided with a significant decrease in expression of PPARGC1A, a key regulator of mitochondrial biogenesis. Down-regulation of PPARGC1A during ccRCC disease progression thus represents a ‘tipping point’ promoting immune dysfunction, cancer progression and poor outcomes from this disease.

Background

In the UK, renal cell carcinoma (RCC) is the fifth most common malignancy in men, and the tenth in women[1]. Risk factors include hypertension, smoking, obesity and end-stage renal disease. A third of cases present with metastatic disease at diagnosis and a third of those undergoing surgery for early-stage disease will relapse, after a median 1.9 years[2]. RCC has joined the growing list of cancers where the PD-1 immune-checkpoint inhibitor nivolumab alone or in combination with ipilimumab[3] has improved overall survival, resulting in a reappraisal of immunotherapy for RCC treatment[4].

RCC is considered an immunogenic tumour with a prominent dysfunctional immune cell infiltrate, unable to control tumour growth[5, 6]. Although effector T-cells enter the tumour microenvironment (TME), their phenotype and function are affected by a complex immunosuppressive network of cancer cells, inflammatory cells, suppressive cytokines and stromal cells. These components drive T-cells to differentiate into ‘exhausted’ T-cells displaying decreased effector cytokine secretion and impaired cytotoxicity causing an inability to control cancer growth[7]. T-cell exhaustion is accompanied by a progressive increase in the expression-level and diversity of inhibitory receptors, including PD-1, LAG3, Tim-3, CD152 (CTLA-4) and TIGIT. In RCC, co-expression of PD-1 and Tim-3 on TILs has been associated with higher stages of the disease and a poor clinical outcome[8, 9].
However, targeted immune checkpoint blockade strategies have only been shown to be effective in a subset of patients (~25% of patients in the case of nivolumab), indicating that factors beyond this inhibitory axis are shaping the immune control of tumours. It is becoming increasingly apparent that the fate and function of T-cells are intrinsically tied to their metabolism and T-cells require the machinery to fulfil their bioenergetic and biosynthetic needs to support proliferation and effector function[10]. Thus, it is unsurprising that T-cells fail in the complex TME of RCC, which can be characterized by loss of function of the tumour suppressor protein von Hippel Lindau (pVHL) resulting in aberrant activation of the hypoxia inducible factors HIF-1α and HIF-2α[11]. The resulting pseudohypoxic phenotype in RCC tumours leads to massive angiogenesis and dysregulated metabolism of the tumour cells themselves limiting nutrients and accumulating immunosuppressive waste products[12]. Thus, the complex signals within the TME promotes effector T-cells with metabolic needs that cannot be met resulting in a loss of tumour immunity.

Recently, Siska PJ et al. investigated the functionality and intrinsic metabolism of ccRCC TILs from a clinically undefined cohort of patients with RCC and found them to be phenotypically distinct and both functionally and metabolically impaired, with the TILs unable to efficiently uptake glucose or perform glycolysis and demonstrating elevated mitochondrial ROS[13].

Here we also investigated the phenotype, function and metabolic capability of TILs from RCC but correlated this with the clinicopathological features of the tumour and the metabolic TME comparing p1-2 to the normal baseline control (normal kidney), p3-4 to stage p1-2 and thrombi and RCCmet to stage p3-4. Consistent with previous studies, RCC CD8 + TILs displayed increased levels of the exhausted phenotype PD1 + CD39 + upon progression into p3-4 advanced stage disease. This coincided with a significant decrease in expression of PPARGC1A, a key regulator of mitochondrial biogenesis, in p3-4 tumours compared to p1-2 tumours. These findings support the critical role that PPARGC1A plays in maintaining CD8 + T-cell fitness and the down-regulation of this gene during ccRCC disease progression represents a ‘tipping-point’ promoting immune dysfunction, cancer progression and poor outcomes from this disease.

Materials And Methods

Human tissue

The study evaluated tumour tissue and blood samples from renal cancer patients from the Royal Marsden Hospital (London, UK) and Frimley Park Hospital (Camberley, UK). Written informed consent was obtained and the donation of tumour tissue and blood samples for evaluation had received Research Ethics Committee approval (study no:12/L0/1661).

Isolation of human peripheral blood mononuclear cells

Whole blood collected in BD Vacutainer blood tubes (BD Biosciences, UK) was diluted 1:1 in RPMI1640 medium and separated by centrifugation on Histopaque. Peripheral blood mononuclear cells (PBMCs)
were harvested from the interface, washed and counted.

**Dissociation of human tumour tissue**

RCC tumour biopsy samples, surplus to diagnostic requirements, were collected immediately following surgical resection and transported cold directly to the laboratory for processing. Tumour biopsies were subjected to a commercial mechanical/enzymatic dissociation system (GentleMACS, Miltenyi Biotec, Germany). After disaggregation, the TILs suspension was passed through 70-µm strainers and treated with red blood cell lysis solution (Qiagen). Following washing the TILs were resuspended in RPMI1640/10%FCS and counted using a haemocytometer.

**Flow cytometry**

Analysis of TILs from RCC tumour and blood samples were performed on cells directly following isolation. The following anti-human antibodies were used: CD45-V450, CD4-APC or CD8-APC, CCR7-PE, CD27-APC-Vio770 (Miltenyi Biotec), PD-1-PECy7 (BD Biosciences), LAG-3-FITC(R&DSystems), TIGIT-FITC (eBioscience), anti-TIM3-PerCPCy5.5 (eBioscience), and BTLA-PerCPCy5.5 (Biolegend). Cells were stained in PBS/1% FCS (Miltenyi Biotec) for 30min at 4°C. JC-1 (2µM) (Molecular Probes), MitoTracker Green (100nM) (Invitrogen), MitoSOX (Invitrogen) and 2-NBDG (Invitrogen) were used per manufacturer’s instructions. Acquisition was performed on a MACSQuant flow cytometer (Miltenyi Biotec) and the MACSQuantify software used for analysis.

**Intracellular cytokine release**

PBMCs were stimulated on anti-CD3 (OKT3) (eBiosciences) coated plates plus soluble anti-CD28 (eBiosciences) overnight. 1µg/ml brefeldinA (Sigma-Aldrich) was added for the last 4hrs of culture. Cells were then harvested and stained for surface markers CD45, CD8, CD4 (Miltenyi) and PD-1 (BD Biosciences). Cells were fixed and permeabilized for detection of intracellular molecules using anti-IFNγ, and TNFα (Miltenyi Biotec). Samples were acquired on a MACSQuant flow cytometer (Miltenyi Biotec) and the MACSQuantify software used for analysis.

**Seahorse Metabolic Assay**

The oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured on an XF Extracellular Flux Analyzer (Seahorse Bioscience). CD3 T-cells were stimulated with 1µg/ml CD3 (OKT3) (eBiosciences) and 20U/ml IL-2 in non-buffered RPMI1640 medium during the assay. Inhibitors were 1µM oligomycin, 1.5µM FCCP, 1µM antimycin A (Sigma Aldrich), and 100nM rotenone (Seahorse Bioscience).

**Total RNA extraction from FFPE-tumour tissues**

Total RNA was isolated from paraffin-embedded tumour tissues using Norgen FFPE RNA Purification kit (Norgen Biotek, Canada) as per the manufacturer’s instructions. The RNA concentration and purity was measured using an Agilent 2100 BioAnalyzer.
Nanostring

Digital multiplexed NanoString nCounter analysis system (NanoString Technologies, USA)-based gene expression profiling was performed on 100ng total RNA from each sample according to the manufacturer’s instructions. Nanostring RNA analysis of 180 metabolism-related human genes was performed using the nCounter GX Human Cancer Metabolism profiling Kit (XT) on the nCounter® Analysis System. Analysis and normalization of the raw Nanostring data was performed using nSolver Analysis Software v1.1 (Nanostring Technologies).

Analysis of the associations between gene expression, overall survival and clinicopathology

TCGA gene expression quantification for ccRCC (TCGA-KIRC) was downloaded from the TCGA repository (https://portal.gdc.cancer.gov/repository, accessed:19th May 2019) consisting of RNA-seq High-Throughput Sequencing count files. Of 538 primary solid tumour samples, 367 are stage p1/2, 241 are stage p3/4 and 3 are ‘not reported’. The edgeR bioconductor package (v3.32.1) was used for data pre-processing {DOI: 10.1093/bioinformatics/btp616}. Lowly expressed genes were filtered out by keeping genes with Counts Per Million (CPM) > 0.129 (median CPM of 8 counts) in at least 25% of samples. Data was normalised using the trimmed mean of M-values normalisation method (TMM). Survival analyses were performed using R packages (Rv4.0.4) survival (v3.2–7) and survminer (v0.4.9) on TMM normalised log2(CPM). Each gene was assessed through a univariate Cox regression model and overall survival Hazard ratios (with 95% confidence interval) calculated using stratified gene expression (high = above 75%, low = below 25%). Kaplan–Meier overall survival analyses of patients stratified according to gene expression (high = above 75%, low = below 25%) were performed and log rank p-values calculated.

Statistical Analysis

Statistical analyses were performed using the non-parametric Mann-Whitney or Wilcoxon matched-pairs test as appropriate, and significant differences are indicated in the figures (*p < 0.05; **p < 0.005; ***p < 0.0005).

Results

Pd1 Expression On Rcc Tils Increases With Stage Of Disease

Firstly, we evaluated PD-1 expression on freshly resected and dissociated RCC tumour cell suspensions from 17 stage p1-2 tumours, 17 stage p3-4 tumours, 9 locally advanced IVC tumour thrombi and 3 distant RCC metastatic sites and compared them to cell suspensions derived from benign cases, normal kidney tissues, blood from RCC patients and blood from healthy controls. As shown in Fig. 1A, PD1 expression on T-cells increased with disease stage with p3-4 RCC tumours, locally advanced tumours and metastatic sites displaying the highest expression of PD-1 on CD4 + and CD8 + T-cells. In comparison, the low level of PD1 expression on T-cells was the same for all the control tissues (benign tumours, normal kidney tissue,
blood from RCC patients and healthy controls). To provide more evidence that these high expressing PD-1+ TILs from the more advanced stage tumours were indicative of exhausted T-cells, in some of the cases CD39 (a marker associated with an exhausted subset of CD8+ T-cells[14]) was combined with PD1 and T-cell markers. CD39 expression on PD-1+ TILs increased with disease stage mirroring the increase in PD-1 expression (Fig. 1B). From 9 of the advanced p3-4 RCC tumours we obtained multiple biopsies taken from different sites within the tumour. Whilst the expression of PD1 on T-cells isolated from different sites within a tumour was largely the same, there were several cases where there was significant heterogeneity in PD1 expression between different biopsy sites (Fig. 1C). Additional inhibitory receptors (TIM3, LAG-3, TIGIT, BTLA) were also investigated on the RCC TILs. There was no significant increase in expression of these receptors on CD4+ T-cells except for TIGIT which was expressed by the majority of TILs from metastatic sites. Notably, the expression of TIM3 was increased on TILs from some of the more advanced disease stages, in particular the RCC metastatic sites (Sup Fig. 1).

**Rcc Tils From Advanced Stage Disease Show Reduced Capability To Produce Th1-cytokines Upon Stimulation**

Single cell suspensions of RCC tumours at different stages of the disease were stimulated *in vitro* to assess the ability of the T-cells to produce IFNγ and TNFα and compared to T-cells from the blood of the patients or healthy controls. As shown in Fig. 2 the CD8+ RCC TILs, from more advanced stages of the disease, showed a significantly reduced capability to produce both IFNγ and TNFα upon stimulation. This contrasted with the CD8+ RCC TILs from early stage (p1-2) RCC tumours which retained their ability to produce Th1-cytokines. The CD4+ RCC TILs followed a similar trend to the CD8+ T-cells but with a lesser impairment to cytokine production in advanced stage disease.

**Impaired Metabolic Function Of Rcc Tils In Advanced Stage Disease**

To understand the functional impairment of RCC TILs during advanced stages of the disease, various assays to measure the metabolic reprogramming of the T-cells, required for effector function, were utilised. First, to examine the capacity for glucose uptake by RCC TILs, we pulsed T-cells with the fluorescent glucose analog 2-NBDG at the end of culture with or without TCR (T-cell receptor) stimulation. Whilst the basal uptake of 2-NBDG by resting PD1+ RCC TILs was similar to resting control T-cells, following stimulation RCC TILs had defects in glucose uptake. This was evident across all stages of RCC but more evident from stage 3–4 and from the locally advanced and metastatic sites (IVC). This contrasted with the T-cells from the blood of RCC patients that were able to increase glucose uptake upon stimulation (Fig. 3).

To further probe the metabolic phenotype of RCC-derived TILs, we measured cellular oxygen consumption rates (OCRs) during a mitochondrial stress test. The spare respiratory capacity (SRC), calculated as change in mean oxygen consumption rate upon treatment with FCCP (fluorocarbonyl cyanide phenylhydrazone), of sorted CD3 + TILs from RCC tumours varied. Most stage 1/2 RCC tumours displayed TILs with a high SRC whilst the TILs from tumours exhibiting invasive morphology ( > =
pT3/IVC thrombus sites) often had a low/minimal SRC. This inability to generate additional energy through oxygen consumption in situations of metabolic stress was specific to TILs from advanced stage cases and not observed in the early-stage tumours or corresponding blood of RCC patients (Fig. 4).

As depleted mitochondrial mass may be one cause of decreased respiration, MitoTracker Green (MTG) staining of RCC-derived PD1+ TILs was carried out. This analysis revealed that whilst the TILs from early stage (p1-2) RCC cases had an equivalent mass to T-cells from the blood of RCC patients, the TILs from advanced stage disease showed a decreased mitochondrial mass (Fig. 5A).

We next investigated mitochondrial function by analysing the production of mitochondrial ROS levels in TILs from RCC patients using the mt-superoxide-specific dye MitoSOX Red and compared them with those of T-cells from the blood of the patients and healthy controls. The mitochondrial superoxide content was similar in unstimulated TILs from all stages of RCC as compared to the T-cells from the blood of patients or healthy controls except for 2 cases of IVC thrombus and a stage 3 tumour which displayed very high levels of basal ROS. Upon anti-CD3 stimulation, superoxide levels consistently increased in T-cells from the blood of control donors and from TILs from stage 1/2 RCC patients, whereas a mixed response with most samples exhibiting a decline or no change of superoxide levels was observed in TILs from stage 3/4 RCC patients and from IVC thrombus and metastatic sites (Fig. 5B).

Further investigation of the mitochondrial function of RCC TILs was carried out by examining the mitochondrial membrane potential (MMP) in freshly isolated TILs from different stages of RCC, after overnight stimulation with anti-CD3 antibody, using the lipophilic cation JC-1. JC-1 is mitochondria selective and forms aggregates in polarized mitochondria that result in a green-orange emission after excitation. However, the monomeric form present in cells with depolarized mitochondrial membranes emits only green fluorescence. After anti-CD3 stimulation, TILs from stage 3–4 RCC patients and from locally advanced (IVC thrombus) and metastatic sites displayed an increased percentage of cells with mitochondrial depolarization as compared to TILs from stage 1–2 RCC patients or T-cells from the peripheral blood of patients or healthy controls (Fig. 5C). This decreased MMP together with the inability to increase ROS levels upon stimulation further supports the existence of dysfunctional depolarized mitochondria within advanced RCC TILs.

Metabolic profiling of the RCC tumour microenvironment reveals metabolic reprogramming from an early stage of disease

To explore whether the observed emergence of T-cell dysfunction in ccRCC at advanced stages of disease is associated with metabolic alterations within the tumour microenvironment a Nanostring nCounter cancer metabolism panel assay was performed on RNA obtained from 30 of the ccRCC cases studied above. Differential gene expression analysis was determined for each stage of the disease (p1-2, p3-4, IVC thrombi and RCCmet) comparing P1-2 to the normal baseline control (normal kidney), P3-4 to stage p1-2 and thrombi and RCCmet to stage P3-4. (Fig. 6). This analysis revealed that even during early stages of the disease (P1-2) there was over-expression of genes (LDHA, SLC2A1, HK2, SLC16A1, HK1) corresponding to proteins associated with glucose metabolism and solute transport functions that could
potentially render the cancer cells more fit whilst at the same time imposing metabolic effects (nutrient deficiency and waste product toxicity) that restrict the TILs. In addition, \textit{PDK1}, a gene known to regulate RCC cell proliferation, migration, invasion and epithelial mesenchymal transition, as well as HIF-responsive genes, \textit{ENO2} and \textit{EGLN3}, were also over-expressed.

Similarly, we observed down-regulation of particular metabolic genes (\textit{ALDOB}, \textit{AC01}, \textit{FBP1}, \textit{EGF}, \textit{SUCLG1} and \textit{PDGFRA}) also from an early (p1-2) stage of disease. These findings were in keeping with previous reports showing that \textit{ALDOB} and \textit{FBP1} were some of the most down-regulated genes in RCC\cite{15, 16}.

Progression into p3-4 stage disease compared to p1-2 and thrombi compared to p3-4 showed an overall down-regulation of metabolic genes. Of note there was a significant down-regulation of \textit{PPARGC1A} in p3-4 stage disease, a gene that encodes peroxisome proliferator-activated receptor gamma coactivator-1, consistent with previous reports of low expression of \textit{PPARGC1A} in ccRCC tissues\cite{17}. In thrombi compared to p3-4 stage disease there was a significant down-regulation of the kidney-specific NADPH oxidase isoform 4 (\textit{NOX4}) which produces considerable amounts of ROS in ccRCC. In the most advanced stage of the disease, the RCCmets compared to p3-4 stage showed an up-regulation of genes (\textit{FASN}, \textit{PFKM}, \textit{PLD1}, \textit{AKT2}, \textit{Citrate synthase (CS)}, \textit{MDH2}) that are associated with aggressive cell proliferation, migration, apoptosis, lipid droplet formation and regulate metabolic disorders of the ccRCC microenvironment. Similar to the thrombi, RCCmet showed a significant down-regulation of \textit{NOX4} along with aldolase B (\textit{ALDOB}) and the glucose solute carrier, \textit{SLC2A2}.

The most significantly up- or down-regulated genes from each stage comparison were then selected for analysis of associations with overall survival (OS) using mRNA expression data from the TCGA database (Sup Fig. 2). For genes differentially expressed in stage p1-2 relative to normal tissue, the results demonstrated that for the up-regulated genes increased mRNA expression level of \textit{PDK1} was associated with improved OS whereas the increased expression of \textit{ENO2} was linked to poor OS. For the down-regulated genes \textit{SUCLG1}, \textit{FBP1}, \textit{AC01} and \textit{ALDOB}, low expression was associated with a poor OS. For the majority of the genes down-regulated at stage 3–4 (\textit{PRKAA1}, \textit{DLST}, \textit{PPARGC1A}, \textit{SUCLA2} and \textit{AKT3}) low expression was significantly associated with a poor OS and only for \textit{SLC2A3} and \textit{FASN} low expression was linked with an improved OS. As published work had shown that \textit{PPARGC1A} was involved in shifting the TME from immune-dominant to metabolic-dominant\cite{19}, we explored further the association of \textit{PPARGC1A} expression and survival within different disease stages (Fig. 7). TCGA data revealed that low expression of this gene is associated with poor survival in all stages of RCC with our own data showing a significant down-regulation of this gene in stage p3-4 tumours compared to stage p1-2 (Fig. 6).

For genes down-regulated in the thrombi relative to stage p3-4, low expression of \textit{FOXO3} was associated with a poor outcome whereas low expression of \textit{G6PD} and \textit{RAC2} was linked to improved OS. For genes differentially expressed in advanced metastatic stage, relative to stage p3-4, high expression of many of the up-regulated genes was associated with improved OS, however, high expression of \textit{FASN} was linked to a poor outcome. Low expression of down-regulated genes \textit{SLC2A2} and \textit{ALDOB} was associated with a poor OS.
Discussion

RCC is a disease characterized by a metabolic signature reflecting its adaptation to hypoxia and bioenergetic needs to sustain cellular proliferation[19]. Growing evidence shows that this metabolic profile is responsible for the dysfunctional immune response of TILs which are abundant in ccRCC[14]. This ‘inflamed’ phenotype of tumour should be responsive to immunotherapies such as immune checkpoint inhibition, yet many patients still do not respond. Ascierto et al.[20] explored the gene expression landscape of PD-L1 + RCCs derived from patients with divergent clinical outcomes after anti-PD-1 therapy and showed that a signature of upregulated metabolic genes was associated with treatment failure in patients with PD-L1 + RCC. Conversely, tumours from responding patients had an upregulated immune gene signature. These findings strongly suggested that the tumour-imposed metabolic effects are responsible for the dysregulated immune response in RCC. The current study set out to confirm the dysregulated immune response in ccRCC, the stage of disease at which T-cells become affected, and any association with tumour stage-specific metabolic gene expression. Here, we show the emergence of exhausted T-cells in more advanced stages (p3-4, IVC thrombus and RCC metastases) of the disease based on their PD-1\textsuperscript{high} and CD39 expression and their reduced ability to produce inflammatory cytokines upon in-vitro stimulation. These exhausted T-cells from advanced stage disease also displayed an overall phenotype of metabolic insufficiency, characterized by mitochondrial alterations and defects in glucose uptake. Since historically the majority of RCC patients receiving immunotherapy will have had more advanced disease the inability of their TILs to respond to immunotherapies is unsurprising given their exhausted and metabolically impaired phenotype.

To create a more permissive environment for T-cell anti-tumour activity it is important to understand the TME factors that are negatively impacting the TILs. Given the wealth of data demonstrating the extensive metabolic reprogramming in ccRCC[21] this study explored the metabolic gene expression profile at different stages of the disease that may explain the emergence of the observed exhausted tumour infiltrating T-cells. Even though T-cells derived from early-stage tumours had shown no features of exhaustion (PD-1\textsuperscript{lo} and CD39\textsuperscript{lo} and functionally produced inflammatory cytokines upon stimulation), p1-2 tumours already had significant over-expression of particular metabolic genes. These included genes involved with solute transport functions such as SLC2A1 which encodes the glucose transporter 1 (GLUT1)[22] and SLC16A1 which encodes a proton-linked monocarboxylate transporter that catalyzes the movement of many monocarboxylates, such as lactate and pyruvate, across the plasma membrane[23]. Genes encoding glycolytic enzymes such as HK1, HK2 and LDHA were also over-expressed. The hexokinases phosphorylate glucose to produce glucose-6-phosphate (G6P), the first step in most glucose metabolism pathways[24] whilst LDHA is necessary for conversion of pyruvate to lactate[25].

Additionally, HIF responsive genes ENO2 and EGLN3 were amongst the highest expressed metabolic genes along with genes such as PDK1 and RAC1 which have been shown to be involved in regulating cell proliferation, migration, invasion and metastasis[26–28]. Besides over-expressed metabolic genes, several down-regulated metabolic genes (AC01, ALDOB, SUCLG1 and FBP1) were identified already in stage p1-2 compared to normal tissue, whose low expression was associated with a poor OS. Indeed,
ALDOB and FBP1 are known to be two of the most down-regulated genes in RCC and both are involved in gluconeogenesis[14, 15]. Fructose-1,6-bisphosphatase 1 (FBP1) inhibits ccRCC progression through two distinct mechanisms: (1) by antagonizing glycolytic flux in renal tubular epithelial cells, thereby inhibiting a potential Warburg effect and (2) by restraining cell proliferation, glycolysis and the pentose phosphate pathway in a catalytic-activity-independent manner, by inhibiting nuclear HIF function via direct interaction with the HIF inhibitory domain. This unique dual function of the FBP1 protein explains its ubiquitous loss in ccRCC[15]. Thus, even at early-stage disease ccRCC cells are establishing an advantage for themselves in outcompeting the TILs for vital nutrients. Furthermore, it has been shown that LDHA-associated lactic acid production can suppress T-cell and NK-cell activation and function[19]. Despite the high expression of these metabolic genes during early-stage disease, their resulting restriction of nutrients and/or effect of metabolic waste products within the TME appears to take time to impact the TILs which only displayed exhaustion at advanced disease stages.8

Interestingly, compared to the early disease stage p1-2, at p3-4 and the locally advanced thrombi stages there was an overall decrease in differentially expressed metabolic genes. Notably, the gene PPARGC1A (also known as PGC1α) was significantly down-regulated in our cohort of p3-4 stage RCC, consistent with published data[29]. PPARGC1A is known to be a key transcriptional coactivator that coordinates mitochondrial biogenesis and oxidative phosphorylation in tumour cells to induce metastasis[30]. Recently Ma et al. observed negative correlations between PPARGC1A expression and tumour grade, clinical stage, and M stage in patients with ccRCC[31]. They showed that in high expressing PPARGC1A ccRCCs, immune-related signaling and epithelial-mesenchymal transition pathways were the most enriched, whilst in low expressing tumours, metabolic pathways were highly enriched. This led the authors to speculate that PPARGC1A is involved in shifting the TME from immune-dominant to metabolic-dominant. Furthermore, they showed that PPARGC1A was negatively correlated with abundances of Tregs and CD8 + T-cells. PGC1α is particularly important for TILs to maintain high mitochondrial activity which promotes CD8 + T-cell fitness, memory formation and anti-tumour immunity[32]. Our data and others[33, 34] have clearly shown that tumour-infiltrating T-cells display an overall phenotype of metabolic insufficiency due to loss of mitochondrial function and mass. This study shows this loss of mitochondrial function in tumour-infiltrating T-cells is most evident at tumour stage p3-4 which correlated with the down-regulated expression of PPARGC1A in ccRCC tumours at this stage. The downregulation of PPARGC1A into stage p3-4 may be the ‘tipping-point’ in RCC disease progression, modulating immune activity in the TME of ccRCC affecting treatment efficacy of immunotherapies in RCC and leading to the dismal outcomes of ccRCC. Whilst no specific drugs activating PGC1α are currently available, strategies to direct the metabolic reprogramming of T-cells such as enforcing expression of PGC1α[33] or 4-1BB costimulation of CD8 + T-cells which engages PGC1α-mediated pathways via activation of p38-MAPK[35], results in enhanced mitochondrial capacity and have been shown to improve the antitumour effects of adoptive cell therapy. Adopting such strategies in combination with current immunotherapy treatments may overcome the immunosuppressive metabolic landscape of the ccRCC microenvironment and increase the proportion of patients benefiting from immunotherapy and/or increase its duration of response.
Declarations

Competing Interests.

- The authors have no relevant financial or non-financial interests to disclose.

Declarations

Ethics Approval.

- This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by an NHS Research Ethics Committee of University (12/LO/1661).

Informed Consent.

- Informed consent was obtained from all individual participants included in the study.

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Author Contributions. –

Nicola Annels and Hardev Pandha contributed to the study conception and design. Material preparation, data collection and analysis were performed by Mick Denyer and Nicola Annels. Tumour samples were acquired by David Nicol, Steve Hazell, Anna Silvanto and Matthew Crockett. TCGA analysis was carried out by Carla Moller-Levet. The first draft of the manuscript was written by Nicola Annels and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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References


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