# Immune Check Point Inhibitors and Their Role in Clear Cell Renal Cell Carcinoma Metabolic Perturbations

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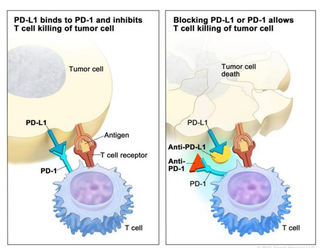
## Abstract

The development of immune checkpoint inhibitors (ICIs) has dramatically increased over the years; however, it remains elusive who will benefit from these therapies and how to monitor their efficacy. We speculated that metabolic monitoring of tumors might be reflective of microenvironment changes induced by ICIs and could indicate their efficacy. It might also improve analytical comparability strategies for prospective biosimilars by better understanding ICIs' biological effects. ICIs are a major treatment modality for patients with advanced clear cell renal cell carcinoma (ccRCC), which features a shift towards aerobic glycolysis. First, we evaluated the metabolic and molecular effect of immune cells on ccRCC cells by performing RNA-sequencing and metabolomic studies on humanized xenograft models and on ccRCC cells treated in-vitro with IFNγ. Both approaches appeared to favor an aerobic glycolytic phenotype, suggesting that immune infiltrates may participate to the metabolic adaptation observed in ccRCC. Next, in-vitro modulation of the immune checkpoint Programmed death-ligand 1 (PD-L1) by transient silencing or using ICIs (avelumab, durvalumab, and atezolizumab), decreased aerobic glycolysis in ccRCC cell lines while concomitantly restoring the metabolic properties of cytotoxic T lymphocytes in co-culture experiments. These in-vitro data suggest that the metabolism of ccRCC cells may reflect ICIs efficacy and provide a rationale for further studies.

## Introduction

A hallmark of many cancers is the dysregulation of cellular energetics and a metabolic shift to increased aerobic glycolysis. Our laboratory investigates how therapeutic antibodies affect the metabolism of clear cell renal cell carcinoma (ccRCC). PD-1 and PD-L1 inhibitors also known as immune check point inhibitors (ICIs) are a group of anticancer drugs that block the activity of PD-1 and PDL1 immune checkpoint proteins present on the surface of cells. Immune checkpoint inhibitors are emerging as a front-line treatment for several types of cancer.

In the cancer disease state, the interaction of PD-L1 on the tumor cells with PD-1 on a T-cell reduces T-cell function signals to prevent the immune system from attacking the tumor cells. Use of an inhibitor that blocks the interaction of PD-L1 with the PD-1 receptor can prevent the cancer from evading the immune system in this way. Several PD-1 and PD-L1 inhibitors are in clinical trial for use in advanced melanoma, non-small cell lung cancer, renal cell carcinoma, bladder cancer and Hodgkin lymphoma, amongst other cancer types. However, the mechanistic roles of ICIs remain largely elusive.




PD-L1 is expressed on tumor cell and PD-1 on T cells. Binding of PD-L1 to PD-1 inhibits killing of tumor cell by T cells. PD-L1 and PD-1 inhibitors block them and allows killing of tumor cell by T cells.

## Results and Discussion

### IFNγ treatment increases PD-L1 expression and increases metabolism in ccRCC cells (A498 and 786-0) which is reversed by PD-L1 silencing

**Figure 1**

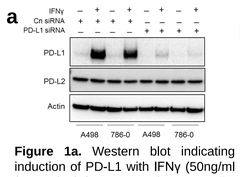



**Figure 1a.** Western blot indicating induction of PD-L1 with IFNγ (50ng/ml for 24 hrs) in A498 and 786-0 cells, this induced PD-L1 expression was silenced by PD-L1 siRNA). Actin expression (housekeeping protein) is used a measure of equal protein loading.

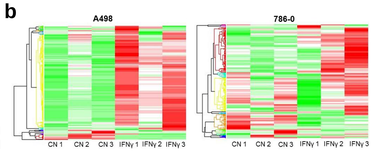



**Figure 1b.** Metabolomic analysis- heat map indicating distinct increase in metabolites with IFNγ treatment in A498 and 786-0 cells as compared to controls. n=3 for each group

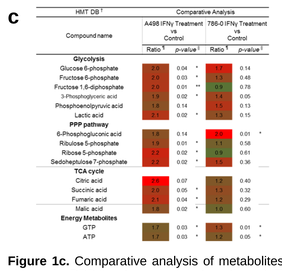



**Figure 1c.** Comparative analysis of metabolites of glycolysis, PPP (pentose phosphate pathway), TCA cycle (tricarboxylic acid), and energy metabolites between IFNγ treated and control A498 and 786-0 cells. †Putative metabolites which were assigned on the basis of m/z and MT in HMT standard compound library. ‡The ratio is computed by using averaged detection values. The latter was used as denominator. ‖The p-value is computed by Welch's t-test (*p<0.05, **p<0.001

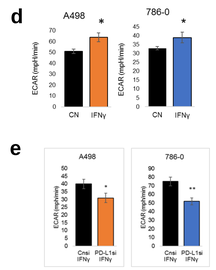



**Figure 1d & e.** Metabolism is decreased in A498 and 786-0 cells with PD-L1 silencing: (d) Basal level ECAR measurement by Extra Cellular Flux Analyzer in IFNγ treated (50 ng/ml for 24 hours) and control A498 and 786-0 cells. The data are presented as mean ± SD (n=3) *p<0.05. (a) Basal ECAR measurement by Extra Cellular Flux Analyzer in IFNγ treated and PD-L1 silenced A498 and 786-0 cells. The data are presented as mean ± SD (n=3) *p<0.05, **p<0.001.

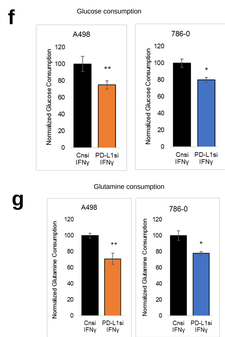



**Figure 1f & g.** A decrease in glucose and glutamine consumption was observed in PD-L1 silenced (PD-L1si IFNγ) A498 and 786-0 cells as compared to IFNγ treated (Cnsi IFNγ) treated cells. The data are presented as mean ± SD (n=3) *p<0.05, **p<0.001.

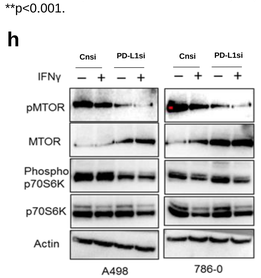



**Figure 1h.** Representative Western blots of MTOR signaling pathway proteins in A498 and 786-0 cells treated with IFNγ and PD-L1 silenced cells. PD-L1 silencing makes ccRCC (A498 and 786-0) cells less glycolytic.

### Inflammatory and Kynurenine pathways are downregulated in ccRCC (A498 and 786-0) cells with silencing of inducible expression of PD-L1

**Figure 2**

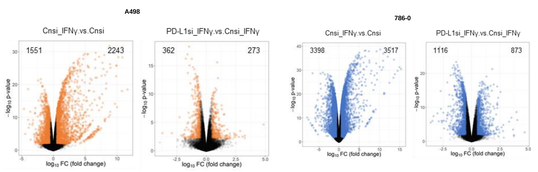



**Figure 2a. RNA-Seq Analysis**: Volcano plots depicting the number of genes upregulated and downregulated in A498 and 786-0 cells with high PD-L1 expression (Cnsi_IFNγ vs Cnsi) and silenced PD-L1 expression (PD-L1si_IFNγ vs Cnsi_IFNγ) (n=3, for each group).

b

| Msig_HallmarkID |
|---|
| INTERFERON_GAMMA_RESPONSE |
| INFLAMMATORY_RESPONSE |
| IL2_STAT5_SIGNALING |
| COMPLEMENT |
| TNFA_SIGNALING_VIA_NFKB |
| KRAS_SIGNALING_UP |
| EPITHELIAL_MESENCHYMAL_TRANSITION |
| PI3K_AKT_MTOR_SIGNALING |
| MTORC1_SIGNALING |

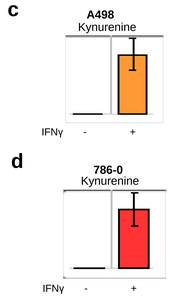
**A498**

| Cnsi IFNγ vs Cnsi | | PD-L1si IFNγ vs Cnsi IFNγ | |
|---|---|---|---|
| NES | pvalue | NES | pvalue |
| 2.5253 | 0.0011 | -1.5798 | 0.0046 |
| 1.9795 | 0.0011 | -1.5866 | 0.0022 |
| 1.6421 | 0.0022 | -1.4727 | 0.0085 |
| 1.9689 | 0.0011 | - | - |
| 1.7243 | 0.0011 | -1.5157 | 0.0045 |
| 1.7507 | 0.0011 | - | - |
| - | - | -1.1960 | 0.1201 |
| - | - | -1.1028 | 0.2795 |
| - | - | -1.5417 | 0.0044 |

**786-0**

| Cnsi IFNγ vs Cnsi | | PD-L1si IFNγ vs Cnsi IFNγ | |
|---|---|---|---|
| NES | pvalue | NES | pvalue |
| 3.1977 | 0.0014 | -2.484 | 0.0019 |
| 2.5079 | 0.0014 | -2.548 | 0.0019 |
| 1.8060 | 0.0014 | -2.022 | 0.0019 |
| 2.1139 | 0.0014 | - | - |
| 2.5861 | 0.0014 | -2.838 | 0.0019 |
| 1.9848 | 0.0014 | - | - |
| - | - | -1.939 | 0.0019 |

**Figure 2b**. GSEA analysis indicating upregulated inflammatory Msig Hallmark pathways with IFNγ in A498 and 786-0 cells are downregulated with silencing of inducible expression of PD-L1.




**A498**

| Gene symbol | Cnsi IFNγ vs Cnsi logFC | Cnsi IFNγ vs Cnsi pvalue | PD-L1si IFNγ vs Cnsi IFNγ logFC | PD-L1si IFNγ vs Cnsi IFNγ pvalue |
|---|---|---|---|---|
| IDO1 | 10.8029 | 3.41E-32 | -0.4201 | 4.64E-08 |
| SLC1A5 | 0.2385 | 1.53E-05 | -0.2800 | 1.62E-06 |
| AFMID | -0.2784 | 0.0020 | 0.0328 | 6.90E-01 |
| KMO | -0.7929 | 4.58E-12 | -0.1162 | 7.39E-02 |
| QPRT | -0.0997 | 0.0662 | 0.2570 | 5.30E-05 |
| NAMPT | 0.6113 | 9.22E-12 | -0.2480 | 3.13E-05 |

**786-0**

| Gene symbol | Cnsi IFNγ vs Cnsi logFC | Cnsi IFNγ vs Cnsi pvalue | PD-L1si IFNγ vs Cnsi IFNγ logFC | PD-L1si IFNγ vs Cnsi IFNγ pvalue |
|---|---|---|---|---|
| IDO1 | 12.0686 | 2.47E-36 | -0.1500 | 4.23E-03 |
| SLC1A5 | 0.3022 | 4.99E-08 | -0.1737 | 2.11E-04 |
| SLC7A5 | 0.2306 | 1.05E-05 | -0.1297 | 5.30E-03 |
| AFMID | -1.0646 | 2.03E-12 | 0.5042 | 1.94E-05 |
| KMO | -0.9892 | 1.25E-18 | 0.6089 | 2.83E-13 |
| QPRT | -1.0483 | 4.92E-17 | 0.4260 | 3.27E-08 |
| NAMPT | 1.9187 | 3.31E-27 | -0.4038 | 1.34E-10 |

**Figure 2 c,d, e and f.** Modulation of kynurenine synthesis pathway with IFNγ treatment and PD-L1 silencing in A498 and 786-0 cells. (**c and d**) Metabolite analysis: IFNγ treatment increases kynurenine oncometabolite as revealed by metabolomics in both 786-0 and A498. The p-value is computed by Welch's t-test, n=3 (**p<0.001). (**e and f**) Differentially expressed genes obtained from RNA-Seq data indicating increase of kynurenine synthesis pathway with IFNγ treatment (Cnsi IFNγ vs Cnsi) that was reversed by silencing inducible expression of PD-L1 (PD-L1si IFNγ vs Cnsi IFNγ) in both A498 and 786-0 cells.

### Silencing the inducible expression of PD-L1 and PD-L1 blockers increase the cytotoxic effect of cytotoxic T lymphocytes (CTLs) on ccRCC cells

**Figure 3**

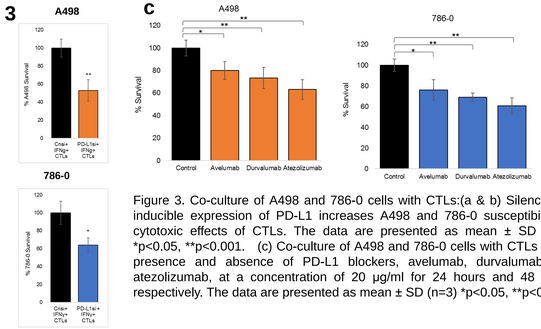



Figure 3. Co-culture of A498 and 786-0 cells with CTLs:(a & b) Silencing of inducible expression of PD-L1 increases A498 and 786-0 susceptibility to cytotoxic effects of CTLs. The data are presented as mean ± SD (n=3) *p<0.05, **p<0.001. (c) Co-culture of A498 and 786-0 cells with CTLs in the presence and absence of PD-L1 blockers, avelumab, durvalumab and atezolizumab, at a concentration of 20 µg/ml for 24 hours and 48 hours respectively. The data are presented as mean ± SD (n=3) *p<0.05, **p<0.001

### PD-L1 blockers by restoring metabolism of CTLs could increase their cytotoxic effect on ccRCC (A498 and 786-0) cells

**Figure 4**

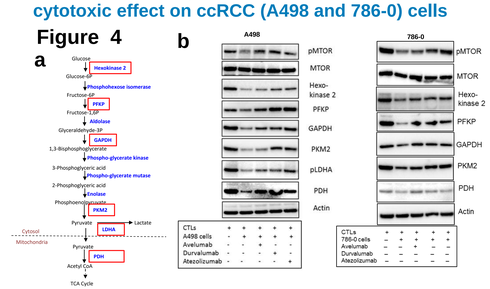



Figure 4. (a and b) Western blot analysis of co-cultured CTLs: CTLs from co-cultured experiments with A498 and 786-0 cells in the presence and absence of PD-L1 blockers were isolated and glycolysis enzymes, PDH (highlighted in red boxes in the pathway) and MTOR were analyzed by Western blots. Mechanistically, PD1-PD-L1 interaction down-regulated AKT-mTOR signaling pathway and glycolysis in CTLs and drives their exhaustion and dysfunction. Blocking the PD1-PD-L1 interaction with ICIs reversed the observed effects, and restored CTLs cytotoxic activity on tumor cells.

## Conclusions

- IFNγ treatment increased the expression of PD-L1 in ccRCC cell lines (A498 and 786-0) and made them more metabolically active. Silencing the inducible expression of PD-L1 in ccRCC cell lines altered the basal metabolism of cells. A decrease in glycolysis and AKT-MTOR signaling pathway was observed indicating modulation of PD-L1 in these cells can cause dysregulation of cellular energetics.
- Silencing the inducible expression of PD-L1 in ccRCC cells reversed the inflammatory and kynurenine pathways upregulated by IFNγ.
- PD-L1 inhibitors increased the cytotoxic effect of CTLs on ccRCC cells and one of the mechanisms could be by increasing AKT-MTOR signaling pathway and glycolysis in CTLs and restoring their activity.
- Our data suggests that silencing of PD-L1 or PD-L1 blockers could make ccRCC less glycolytic and increase the cytotoxic effect of CTLs by restoring their metabolic properties and improving the efficacy of immunotherapies in ccRCC.

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## Disclaimer

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