Understanding the tumor microenvironment to overcome resistance in kidney cancer

Marisol Mosqueda Arreola[1], Arnold H. Zea, PhD[2,3]

[1]LSUHSC School of Medicine, [2]Stanley S. Scott Cancer Center, [3]Department of Microbiology

Introduction

- Renal cell carcinoma (RCC) remains one of the most resistant tumors to systemic chemotherapy, radiotherapy, and immunotherapy.
- When L-arginine (L-arg) is metabolized by arginase (ARG2), it allows the synthesis of polyamines and thus tumor growth.
- However, L-arg can also be converted by NOS2 to nitric oxide (NO) and L-citrulline (L-cit). NO is known to have anti-tumor properties, but L-cit's role is still unknown.
- Furthermore, L-cit can be converted de-novo to L-arg by argininosuccinate synthase (ASS) and lyase (ASL) enzymes (***Figure1***).

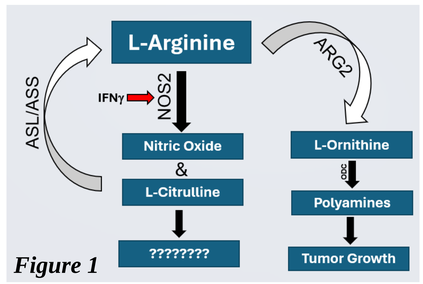

Figure 1

- We have previously demonstrated that:
  - in some RCC, stimulation with INFγ induced NO production which inhibited their proliferation, but some cells were resistant
  - cells lacking NOS2 did not presented with an anti-tumor affect after INFγ stimulation
- These results suggested the direct role of L-arg metabolites in anti-tumor activity and resistance.
- While the schematic above may seem like a straightforward interconnected cycle, the intricacies of how these pathways work are still poorly understood.

Hypothesis

- First, our main goal is to elucidate the associated mechanisms by which certain RCC tumors become resistant to treatment.
- We *hypothesize* that L-citrulline via ASS/ASL enzymes restores L-arginine availability increasing anti-tumor activity via IFNγ-NOS2 signaling dependent on tumor heterogeneity.

Methods

Different murine renal cell carcinoma cells, expressing different ARG2 levels were used.

- Cells were cultured in RPMI media containing 1040 μM of L-arg for 24, 48 and 72 hours.
- Cells were stimulated with IFNγ (100U/ML) or LPS (1μg/ML) or combination for 48 hours.
- Culture supernatants were tested for L-arg and L-cit by HPLC.
- Greiss assay was used to detect nitrite levels.
- Cellular extracts were prepared with Tritox-X100 based lysis buffer for ARG2 and NOS2 protein expression analysis via Western Blot.
- Cell proliferation was measured using MTT assay.
- Data analyses were performed using the GraphPad software Prism 5.0. The non-parametric Student t-test was used. $p < 0.05$ were significant

Stimulated ARG2, NOS2 & Nitrites

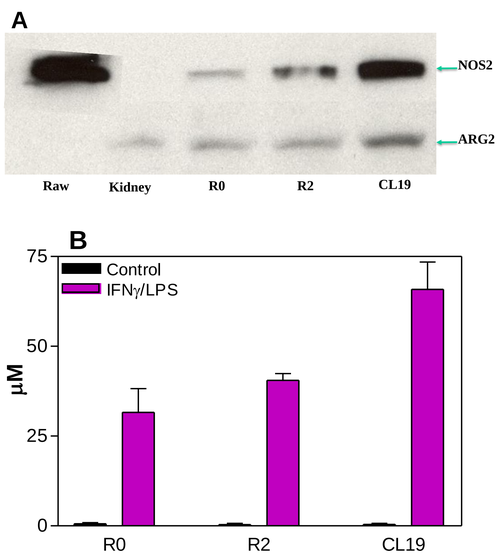

Figure 2. (A) The expression of ARG2 in the 3 cell lines did not change after 48 h stimulation with IFNγ/LPS when compared to the normal kidney. NOS2 expression was different in each cell line, with CL19 having the highest. (B) The results observed by Western blot paralleled with the accumulation of nitrites in the supernatants of the stimulated cells. Differences in nitrite production between non-stimulated and stimulated cells are remarkable.

Effect of IFNγ/LPS on Cell Growth

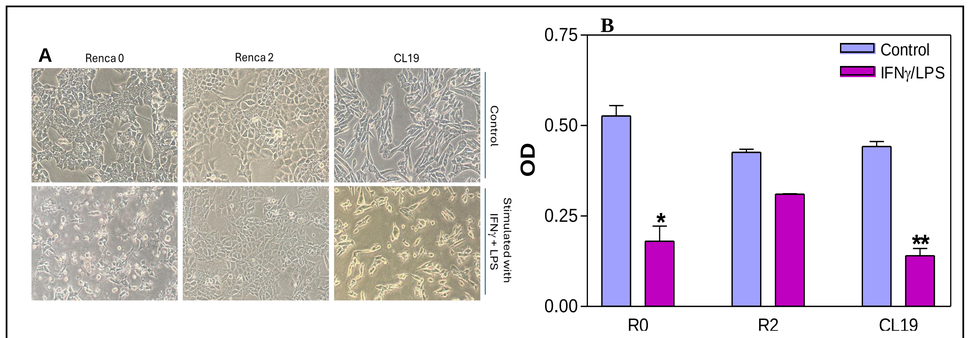

Figure 3. (A) The growth of stimulated Renca0 and CL19 cells was diminished when compared to the unstimulated counterparts. (B) IFNγ/LPS treatment significantly inhibits cell grow in Renca0 (*p= 0.003) and CL19 (**p=0.0005). In contrast, there is not a significant reduction in the growth of the R2 cell line. Data in B is representative of 3 different experiments.

L-arginine & L-citrulline Levels

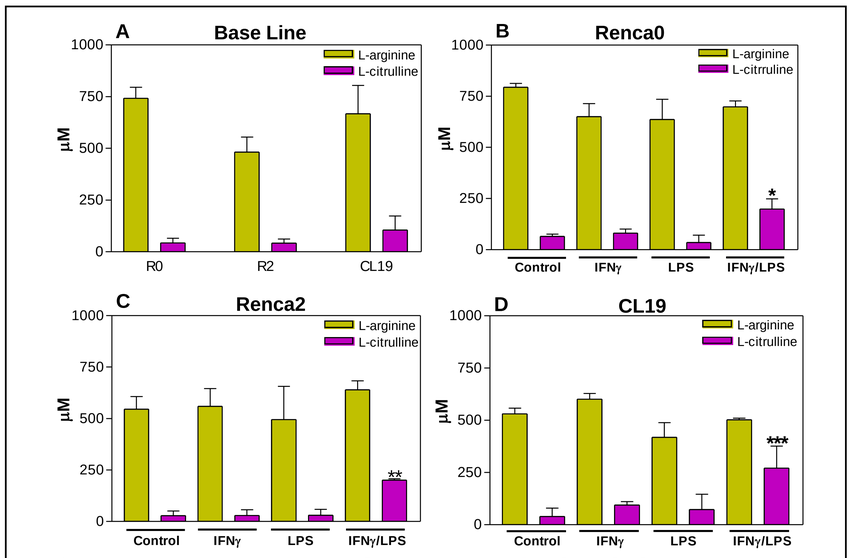

Figure 4. L-arg (gold) and L-cit (purple) levels by HPLC. (A) Base line levels of L-arg range between 550-750 μM among the 3 cell lines and L-cit between 0-75 μM at 48h in culture. (D) There is a significant decrease in L-arg availability and increase in L-cit in CL19 cells (***p= 0.007). (B) There is an increase in L-cit in R0 (*p=0.034) and 50% cell death. (C) Although the R2 cells showed an increase in L-cit (**p= 0.0018), these cells were resistant to this treatment.

Conclusions

- Overall, each cell line showed different sensitivities to IFNγ/LPS stimulation.
- NOS2 expression appears to be independent of Arginase expression. However, we don't know yet if the activity of Arginase is more important than just its expression.
- Because R2 did not show decreased cell death after IFNγ/LPS stimulation, there might be another pathway that needs to be investigated.
- Also, we need to determine the kinetics of L-citrulline toxicity on the studied RCC cells.

Future Plans

- We need to determine whether inhibition is dependent of NO by inhibiting NOS2 activity.
- Because of our hypothesis, we need to determine the role of ASS/ASL in the de novo synthesis of L-arginine and its role in overcoming resistance.
- Because we are focused on the tumor microenvironment and how to stop tumor growth, we cannot avoid the potential effect that INFγ/LPS combination could have in potentiating tumoricidal activity on innate and adaptive immunity and checkpoint inhibitors.