

Research Paper

## Interferon-Gamma-Induced Nitric Oxide Inhibits the Proliferation of Murine Renal Cell Carcinoma Cells

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### Abstract

Renal cell carcinoma (RCC) remains one of the most resistant tumors to systemic chemotherapy, radiotherapy, and immunotherapy. Despite great progress in understanding the basic biology of RCC, the rate of responses in animal models and clinical trials using interferons (IFNs) has not improved significantly. It is likely that the lack of responses can be due to the tumor's ability to develop tumor escape strategies. Currently, the use of targeted therapies has improved the clinical outcomes of patients with RCC and is associated with an increase of Th1-cytokine responses (IFN $\gamma$ ), indicating the importance of IFN $\gamma$  in inhibiting tumor proliferation. Thus, the present study was designed to investigate a new mechanism by which IFN $\gamma$  mediates direct anti-proliferative effects against murine renal cell carcinoma cell lines. When cultured RCC cell lines were exposed to murine recombinant IFN $\gamma$ , a dose dependent growth inhibition in CL-2 and CL-19 cells was observed; this effect was not observed in Renca cells. Growth inhibition in CL-2 and CL-19 cell lines was associated with the intracellular induction of nitric oxide synthase (iNOS) protein, resulting in a sustained elevation of nitric oxide (NO) and citrulline, and a decrease in arginase activity. The inhibition of cell proliferation appears to be due to an arrest in the cell cycle. The results indicate that in certain RCC cell lines, IFN $\gamma$  modulates L-arginine metabolism by shifting from arginase to iNOS activity, thereby developing a potent inhibitory mechanism to encumber tumor cell proliferation and survival. Elucidating the cellular events triggered by IFN $\gamma$  in murine RCC cell lines will permit anti-tumor effects to be exploited in the development of new combination therapies that interfere with L-arginine metabolism to effectively combat RCC in patients.

Key words: nitric oxide, Interferon-gamma, nitric oxide synthase, renal cell carcinoma, arginase 2, polyamines, L-arginine, cell proliferation.

### INTRODUCTION

Renal cell carcinoma (RCC) accounts for 5% of all cancers, leading to an estimate of 13,120 mortalities each year in the United States (1). RCC remains one of the most resistant tumors to immunotherapy, chem-

otherapy, and radiotherapy (2). The overall survival for all RCC patients has not appreciably improved during the last 25 years, indicating the need for more effective ways to treat this disease. Immunotherapy

with interferon alpha (IFN $\alpha$  and/or interleukin-2 (IL-2)) has been used as a first-line therapy to treat RCC, but the documented clinical response rate in patients is only 10-20%, with a median survival of approximately 12 months (3-5). In recent years, the advent of targeted therapies for metastatic RCC has grown substantially (6-9); however, these therapies do not produce complete responses and have several adverse effects, and their mechanisms of action are poorly understood.

There is a complex relationship between immunity, tumorigenesis, and the metabolism of L-arginine (10;11) that can be highly relevant to RCC. Recent studies have demonstrated convincingly that the enzymes arginase 1 (ARG1), arginase 2 (ARG2), and inducible nitric oxide synthase (iNOS) play a crucial role in tumor biology (12-14). ARG1 and ARG2 are collectively referred to as ARG in this manuscript because their activities are similar. L-arginine is metabolized by ARG, producing L-ornithine, as substrate for ornithine decarboxylase (ODC) to synthesize polyamines that are essential for cell growth. In contrast, iNOS metabolizes L-arginine to produce nitric oxide (NO) that kills tumor cells by mechanisms that are partially understood (15-17). Since ARG and iNOS compete for L-arginine, this amino acid is a crucial component whose metabolism modulates cell growth/inhibition and suppresses T-cell function (18). In addition, the balance between iNOS and ARG is competitively regulated by Th1 and Th2 type-cytokines; Th1 cytokines induce iNOS, whereas Th2 cytokines induce ARG (19). One of the possible mechanisms to explain the impairment of antitumor immunity in patients with RCC is the well documented shift from Th1 to Th2 cytokine response (20). This shift may prompt the tumor microenvironment to produce high levels of ARG and polyamines instead of initiating an iNOS-dependent production of NO that could be characterized by the lack of Th1 responses in order to survive (21;22). Recently, we demonstrated that murine RCC cell lines with high levels of ARG2 expression are associated with L-arginine depletion resulting in induced T-cell dysfunction and increased tumor growth (23).

For many years, treatment with IFN $\gamma$  has shown minimal or no activity in patients with RCC. Since the combination of IFN $\gamma$  and IFN $\alpha$  treatment reflected a response rate similar to IFN $\alpha$  alone, the latter has been the treatment of election for RCC patients for many years (24). This observation is striking since IFN $\gamma$  has the most potent immunomodulatory activity among the interferons (25-27), indicating that the mechanisms of action of IFN $\gamma$  are complex, not well understood, and deserve to be re-visited in the context

of tumor resistance and tumor microenvironment.

The present study focuses on the mechanisms by which IFN $\gamma$  can modulate L-arginine metabolism by shifting ARG-dependent cell proliferation to growth inhibition by inducing iNOS and NO production. Understanding these mechanisms will facilitate the development of new, effective, pharmacological strategies that interfere with L-arginine metabolism to control and eliminate RCC in affected patients.

## MATERIALS AND METHODS

### Cell lines, reagents and culture conditions

For this study, three murine RCC (mRCC) cell lines were used: SIRCC-1.2 (CL-2) and SIRCC 1.19 (CL-19) cells of induced origin (28) and Renca cells of spontaneous origin, kindly provided by Dr. Robert H. Wiltout (NCI). Cells were maintained at 37°C in continuous cell culture in RPMI-1640 containing 1,140  $\mu$ M L-arginine (Lonza Inc., Walkersville, MD) and supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 25 mM HEPES, 4 mM L-glutamine, 100 units/ml of penicillin/streptomycin, 1 mM non-essential amino acids, and 1 mM sodium pyruvate (Lonza, Inc.). Recombinant mouse IFN $\gamma$  (rmIFN $\gamma$ ) purchased from R&D Systems, Minneapolis, MN was used to determine its effect on iNOS induction and cytostasis on mRCC cells (10U/ml). The inhibitor of iNOS, N<sup>G</sup>-monomethyl-L-arginine (L-NMMA) (EMD-Calbiochem, Gibbstown, NJ), was also titrated to determine the optimal inhibitory conditions (1mM). The experiments were performed by plating  $3 \times 10^5$  cells in six-well plates which were allowed to attach for 24 h (this cell concentration allow the cells to survive in culture up to 120 h). Fresh media containing IFN $\gamma$  and L-NMMA (Time 0) were added to the cells and cultured at 2, 4, 6, 24, 48, and 72 h. At the different time points, supernatants were collected and cells were harvested using 0.05% Trypsin/EDTA (Sigma, St. Louis, MO) to perform the different assays. The protein concentration was determined by using the BCA protein assay kit (Pierce Biotechnology Inc., Rockford, IL). Cell extracts were stored at -70 °C until used for western blots.

### Arginase activity

Freshly prepared cytoplasmic extracts from cultured mRCC cells before and after treatment with IFN $\gamma$  were tested for ARG activity by the conversion of L-arginine to L-ornithine (nanomoles/10<sup>6</sup> cells/hour), as described elsewhere (29).

### Western blot

Equal amounts of cell extract protein (25

µg/lane) were electrophoresed in 8% Tris-glycine gels (Invitrogen, Carlsbad, CA) and transferred to polyvinylidene difluoride (PVDF) membranes (Invitrogen). Immunoblotting was performed with antibodies for ARG2 (1:500), iNOS (1:200) (Santa Cruz Biotech, Santa Cruz, CA), and GAPDH (1:10,000) (Fitzgerald Industries International, Acton, MA). Detection was achieved by horseradish peroxidase-conjugated antibodies (1:3000, Santa Cruz) and an enhanced chemiluminescent kit (ECL, GE Healthcare, Piscataway, NJ). ARG2 and iNOS protein bands were visualized on X-OMAT AR films (Kodak, Rochester, NY).

### Reverse Transcription-PCR analysis

Total RNA from  $1 \times 10^6$  cells was extracted using TRIzol (Invitrogen), treated with DNase I (Invitrogen), and reverse transcribed using Superscript II (Invitrogen). PCR amplification was completed using primers for mouse iNOS and  $\beta$ -actin as follow: iNOS: forward, 5'-CTT CCG AAG TTT CTG GCA GCA GCG-3', reverse, 5'-GAG CCT CGT GGC TTT GGG CTC CTC-3', and  $\beta$ -actin forward 5'-CCA GAG CAA GAG AGG TAT CC-3', reverse 5'-CTG TGG TGG TGA AGC TGT AG-3'. The expected sizes of amplified fragments were iNOS 459 bp, and  $\beta$ -actin 436 bp. PCR products were visualized in ethidium bromide 1% agarose gels.

### Assessment for cell growth and survival

Cell growth and viability was determined by using the previously described MTT assay (30). Briefly, different cell lines were plated at  $1 \times 10^4$ /well in 24-well plates and treated with IFN $\gamma$ . At 24, 48, and 72 h, cells were incubated with MTT (0.5 mg/ml) for 4 h at 37 °C. Then, an equal volume of 0.04 M HCl in isopropanol was added to the wells and incubated for 3 h to dissolve the accumulated crystals of formazan. Absorbance was measured at 570 nm with a reference wavelength of 650 nm using a Benchmark Plus plate reader (Bio-Rad, Hercules, CA). Readings obtained from treated cells were compared with untreated controls in triplicate.

### DNA fragmentation

Cell lines were treated with IFN $\gamma$  or untreated for 24, 48, and 72 h. At each time point, the cells were harvested by trypsinization and washed once with phosphate buffered saline (PBS). Fragmented DNA was isolated using a DNA isolation kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Eluted samples containing DNA were electrophoresed on a 2% agarose gels (Life Technologies, Grand Island, NY) containing 0.5 µg/ml solution of ethidium bromide. DNA bands were visualized under

UV and exposed to photography.

### Cell cycle analysis

For DNA content analysis,  $1 \times 10^6$  cells were treated with IFN $\gamma$  for 24 and 48 h. The cells were harvested by trypsin digestion, collected by centrifugation, and fixed in 70% ethanol /phosphate buffered saline for at least 12 h at 4°C. After 100 µL (1 g/L) of RNase treatment, cells were stained with 0.5 µg/ml propidium iodide. Cells were examined by flow cytometry using a LSR II (Becton-Dickinson, USA). The percentage of cells in each phase of the cell cycle was analyzed using Modfit software (Verity, Software House, Topsham, ME).

### Determination of Nitric oxide production

Nitrite accumulation in cell culture medium under defined conditions was used as an indirect determination of NO production and measured by a colorimetric assay based on the Griess reaction. Briefly, 200 µL of the culture supernatants were mixed with 200 µL of Griess reagent [one part 0.1% N-(1-naphthyl) ethylene-diamine dihydrochloride and one part 1% sulfanilamide] in 5% concentrated H<sub>3</sub>PO<sub>4</sub> for 10 min at room temperature. The absorbance was measured at 540 nm in a Benchmark Plus plate reader (Bio-Rad). The readings were compared to a standard curve for sodium nitrite with a lower detection limit of 1 µmol/L nitrite. In order to confirm that the formed nitrites were derived from iNOS induction, cells were cultured in the presence of the iNOS inhibitor L-NMMA.

### Amino acid detection

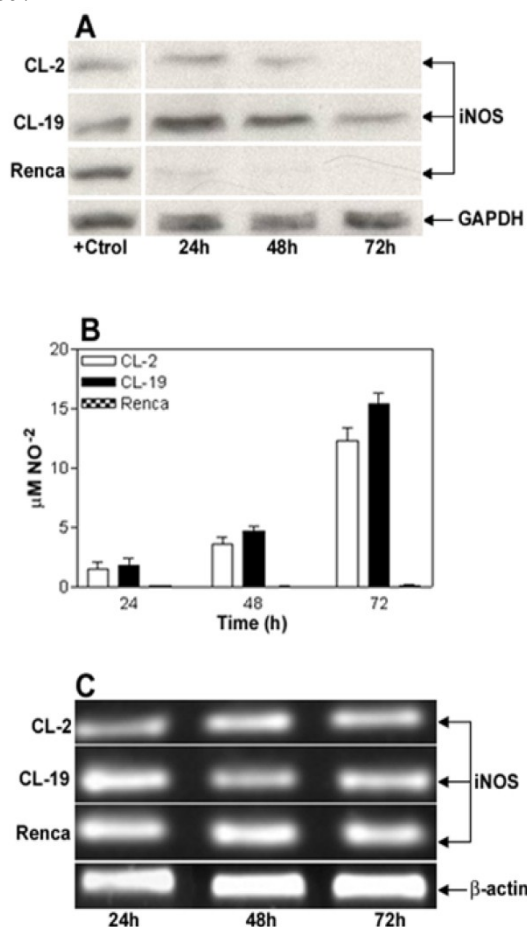
High performance liquid chromatography (HPLC) was conducted on deproteinized conditioned media labeled with *O*-phtaldialdehyde (OPA). Analytes were eluted with 100 mM sodium acetate buffer, pH 5.0, with a linear gradient consisting of methanol (100%) and acetonitrile (100%). The analytes in the samples were calculated on the basis of standard curves of known amounts.

### Cytokine measurement

Cytokines were measured in tissue culture supernatants released from cells at the different time points using Th1/Th2 panel Bio-Plex cytokine assay (Bio-Rad Laboratories, Hercules, CA) as described previously following the manufacturer's instructions (31). The samples were analyzed for IL-2, IL-4, IL-5, IL-10, IL-12 (p70), GM-CSF, IFN $\gamma$ , TNF $\alpha$ , IL1 $\beta$ , IL6, and VEGF on a Bio-Rad 96-well plate reader using the Bio-Plex Suspension Array System and Bio-Plex Manager software (Bio-Rad).

## Statistical analysis

Statistical analyses were completed in SAS v9.1 (SAS Institute, Cary, NC). For each cell line, nitrite concentration, proliferation, and ARG activity data were analyzed by mixed models for unequal variances. Two-way ANOVA models were used with time and experimental conditions (basal/ $\text{IFN}\gamma$ , control/ $\text{IFN}\gamma$ , and  $\text{IFN}\gamma$ +LNMMA) as factors. After  $\text{IFN}\gamma$  treatment, each experimental condition was tested at each time point. Multiple testing was conducted at the 5% level using the Tukey procedure. Least squares means and corresponding standard errors were obtained.



**Figure 1. Characterization of iNOS expression and iNOS activity in murine RCC cell lines cultured in normal conditions.** (A) Representative Western blot analysis for iNOS (130 kDa) and GAPDH (35 kDa) in CL-2, CL-19 and Renca cells cultured in normal conditions for 24, 48, and 72 h. Twenty five µg of total protein was loaded per line. RAW 264.7 cells stimulated with LPS plus murine  $\text{IFN}\gamma$  were used as positive control (+Ctrl) for iNOS protein. (B) Nitrite production in the 3 cell lines was determined by Greiss reaction in culture media supernatants collected after 24, 48, and 72 h in culture. Results are the mean  $\pm$  SE of triplicates of five different separate experiments. (C) Reverse transcription-polymerase chain analysis of iNOS mRNA expression. The result of a representative experiment is shown; similar results were obtained in at least three experiments. The expected sizes of amplified fragments were 459 bp for iNOS and 436 bp for  $\beta$ -actin.

## RESULTS

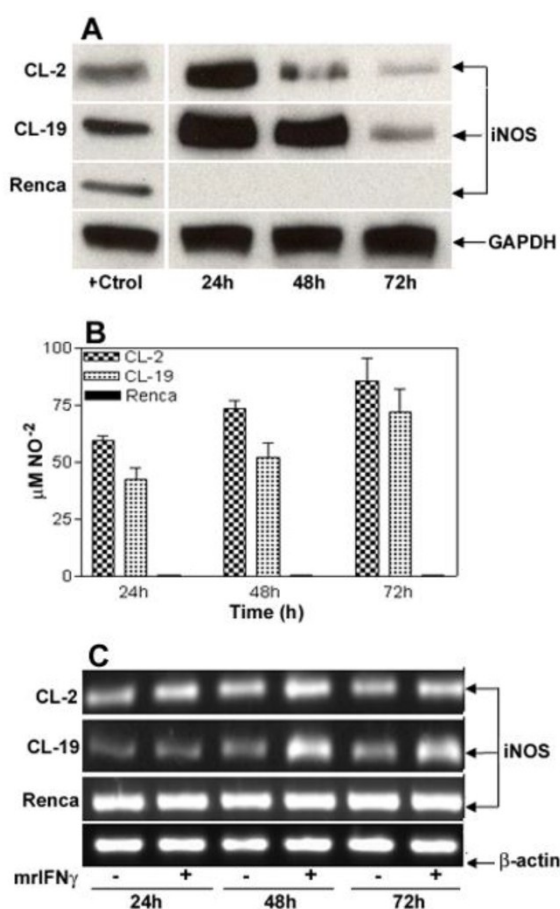
### iNOS protein expression and NO production differs in mRCC cell lines

The basal expression level of iNOS protein and nitrite production in RCC cell lines CL-2, CL-19, and Renca, cultured in complete RPMI media for 24, 48, and 72 h was determined. As shown in Figure 1A, iNOS protein is detected at moderate levels in CL-2 and CL-19 during the time course of this experiment. In contrast, a complete absence of iNOS protein expression was observed in Renca cells. When supernatants were tested for NO release, a minimal and continued synthesis of nitrites ranging from  $1.4 \pm 0.64$  µM at 24 h to  $12.2 \pm 1.12$  µM at 72 h in the CL-2 cells and from  $1.7 \pm 0.63$  µM up to  $15.4 \pm 0.86$  µM in CL-19 cells was observed. Although low levels of iNOS are expressed in CL-2 and CL-19 in the absence of  $\text{IFN}\gamma$ , we were unable to detect nitrites released by iNOS activity at the level of detection available to us, before 24 h. However, production of NO was not observed in Renca cells at any tested time point (Figure 1B). The absence of iNOS protein expression in Renca cells prompted us to test iNOS mRNA expression by reverse-transcriptase PCR in the aforementioned cell lines. As shown in Figure 1C, iNOS mRNA gene expression was similar in all three cell lines at the different time points of the experiments.

### $\text{IFN}\gamma$ increases iNOS protein levels and NO production in CL-2 and CL-19 but not in Renca cells

To determine the level of toxicity of  $\text{IFN}\gamma$  on the cell lines, kinetic experiments using concentrations of  $\text{IFN}\gamma$  ranging from 2.5 to 500 U/ml were performed (data not shown). In this system, it was observed that 10 U/ml of  $\text{IFN}\gamma$  was the optimal concentration to induce iNOS expression and to stimulate NO production without a measurable toxic effect on the cells.  $\text{IFN}\gamma$  concentrations higher than 15 U/ml were toxic to CL-2 and CL-19 cell lines, where a complete detachment of the cell monolayer was observed as early as 12 h (data not shown). Immunoblot analysis showed a maximal increase in the expression of immunoreactive iNOS protein in CL-2 and CL-19 cells at 24 hours, post-treatment. iNOS levels started to decrease at 48 h and reached basal levels at 72 h (Figure 2A). Interestingly,  $\text{IFN}\gamma$  did not induce iNOS protein or cytostasis in Renca cells. This finding prompted us to examine iNOS gene expression pre- and post-  $\text{IFN}\gamma$  treatment. Strikingly, iNOS mRNA was expressed in Renca cells at the same levels before and after treatment; this finding suggests a post-transcriptional

regulation of iNOS expression. In contrast, IFN $\gamma$  treatment markedly enhanced levels of iNOS mRNA in CL-2 and CL-19 cell lines (Figure 2B). The increased iNOS mRNA and protein expressions in CL-2 and CL-19 cells are associated with the production of high levels of NO ranging from  $59.7 \pm 2.0 \mu\text{M}$  at 24 h to  $85.6 \pm 9.6 \mu\text{M}$  at 72 h in CL-2 cells and from  $42.4 \pm 5.3 \mu\text{M}$  at 24 h to  $71.9 \pm 9.9 \mu\text{M}$  at 72 h in CL-19 cells (Table 1). As expected, production of NO was not observed in Renca cells throughout the course of the experiments. Minimum iNOS protein expression was observed in untreated cells (Figure 1A) that were used as control against treated cells.



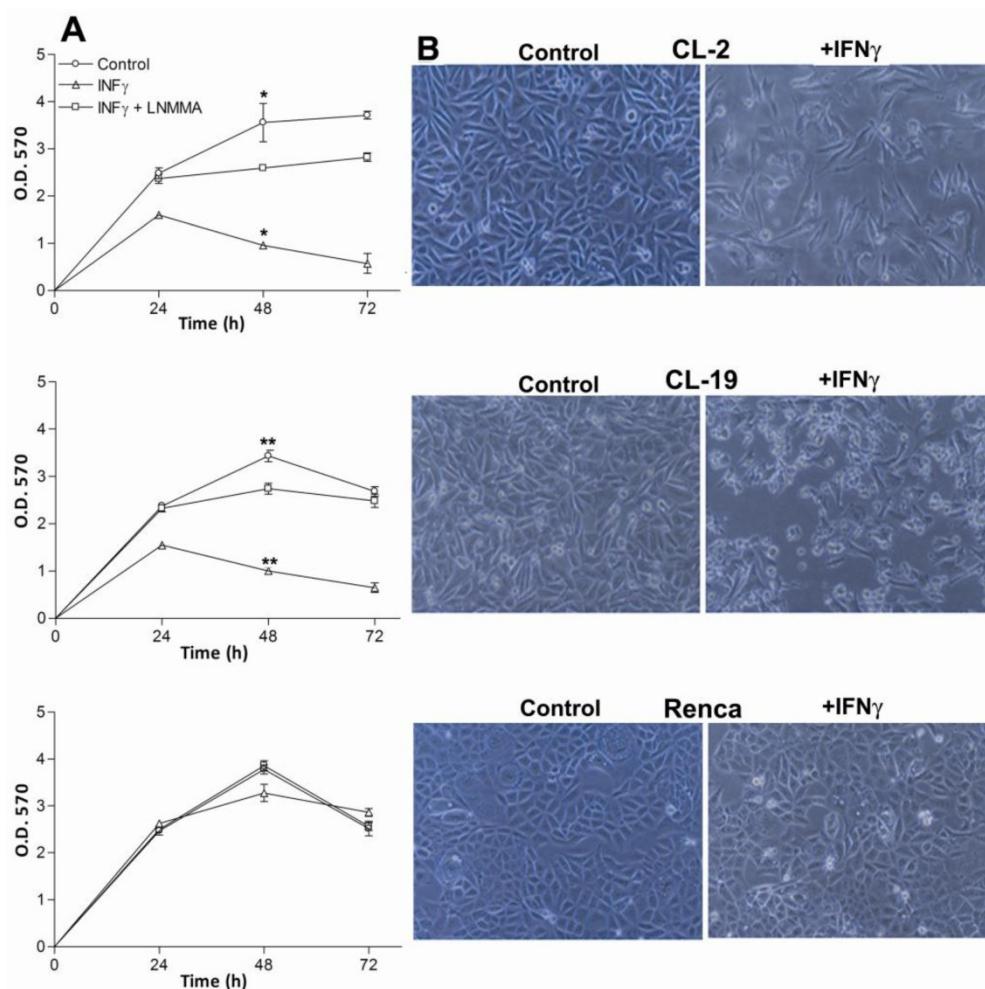
**Figure 2. IFN $\gamma$ -induced iNOS expression and iNOS activity in murine RCC cell lines.** For reference, Figure 1A can be used as untreated control for these experiments. **(A)** Time course of iNOS protein expression by Western blot after stimulation of cells for 24, 48, and 72 h with 10 U/ml of murine IFN $\gamma$ . **(B)** Nitrite production in culture supernatants following stimulation with IFN $\gamma$ . Values for nitrite production are expressed by mean  $\pm$  SE for three different experiments performed five different times. **(C)** Pattern of 24, 48, and 72 h iNOS mRNA induction (RT-PCR) in response to IFN $\gamma$  stimulation. Each Western blot and RT-PCR presented is from single experiment that is representative of three to five separate experiments.

**Table 1. Nitrite concentration ( $\mu\text{M}$ ) in RCC cell lines of NO $^{2-}$  before and after treatment with IFN $\gamma$ .** <sup>1</sup> Means of 6 different experiments; <sup>2</sup> SE; \* p-values for significance ( $p=0.0001$ ) between untreated and treated cells with IFN $\gamma$  for each cell line.

| Time | CL-2                                     |                  | CL-19            |                  | Renca         |               |
|------|--|------------------|------------------|------------------|---------------|---------------|
|      | -IFN $\gamma$                            | +IFN $\gamma$    | -IFN $\gamma$    | +IFN $\gamma$    | -IFN $\gamma$ | +IFN $\gamma$ |
| 24h  | 1.4 <sup>1</sup> $\pm$ 0.64 <sup>2</sup> | 59.7 $\pm$ 2.01  | 1.7 $\pm$ 0.63   | 42.4 $\pm$ 5.29  | 0             | 0             |
| 48h  | 3.5 $\pm$ 0.61                           | 73.3 $\pm$ 3.79  | 4.6 $\pm$ 0.36   | 52.0 $\pm$ 6.69  | 0             | 0             |
| 72h  | 12.2 $\pm$ 1.12*                         | 85.6 $\pm$ 9.62* | 15.4 $\pm$ 0.86* | 71.9 $\pm$ 9.93* | 0             | 0             |

### IFN $\gamma$ induces NO-mediated cell death in CL-2 and CL-19 cell lines that is abrogated by the iNOS inhibitor L-NMMA

The effectiveness of IFN $\gamma$  in inhibiting the proliferation of mRCC cell lines was assessed using MTT assays. As seen in Figure 3A, the addition of 10U/ml of IFN $\gamma$  to mRCC cell lines significantly inhibited cell proliferation of CL-2 ( $p=0.008$ ) and CL-19 ( $p=0.0001$ ) after 48 h in culture when compared to untreated cells. Not surprisingly, no significant difference in proliferation ( $p=0.091$ ) was observed in Renca cells. As seen in Figure 3B, differences in cell morphology and cell growth in CL-2 and CL-19 treated cells were observed at 48 hours post-treatment; these effects were absent in Renca cells at the same time-point. To test whether IFN $\gamma$  inhibited cell proliferation and induced cell death through the endogenous production of NO, L-NMMA, a non-selective inhibitor of all NOS isoforms, was added to the cultures. Confirming the cell death resulting from NO production, L-NMMA completely reversed the killing effects of IFN $\gamma$  treatment (Figure 3A). NO production was also measured directly from the supernatants and showed that LNMMA completely inhibited the production of nitrites after stimulation with IFN $\gamma$  in CL-2 and CL-19 cell lines ( $*** p<0.0001$ ) as seen in Figure 4. The results are most easily interpreted to indicate that the endogenous production of NO by IFN $\gamma$  treatment has a potent inhibitory effect on tumor cell survival and proliferation. Only endogenous NO inhibited cell proliferation and survival because control experiments, where nitrites were added exogenously to the media, showed no effect on cell survival (data not shown).



**Figure 3. Effect of IFN $\gamma$  and LNMMA on CL-2, CL-19 and Renca cell proliferation. (A)** Three hundred thousand cells from CL-2, CL-19 and Renca were seeded in six well plates and cultured in presence or absence of IFN $\gamma$  (10U/ml) or iNOS inhibitor LNMMA (1mM). Cell proliferation was measured at 24, 48, and 72 h using the MTT assay. The results are expressed as O.D (570) means  $\pm$  SE of triplicate determinations from five independent experiments. Significant differences in proliferation were observed between control and IFN $\gamma$  treated CL-2 (\*  $p=0.0084$ ) and CL-19 (\*\* $p=0.0001$ ) cells respectively. **(B)** Morphological changes and inhibition of cell proliferation in the CL-2, CL-19 and Renca cells before and after treatment with IFN $\gamma$ . Microscopic images of mRCC cells after treatment with IFN $\gamma$  were taken at 48h. Photographs were taken under phase contrast at 100x magnification.

### Effects of IFN $\gamma$ on DNA fragmentation and cell cycle in the cell lines

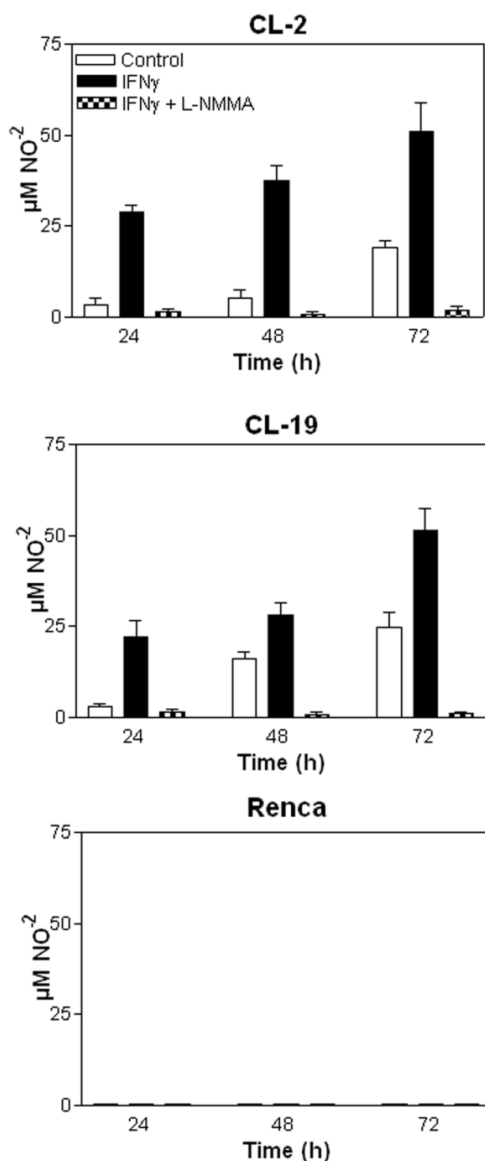
Apoptosis is characterized by DNA fragmentation; therefore, DNA was isolated from the three cell lines after treatment with IFN $\gamma$  at 24, 48, and 72 h and tested for its integrity. The results shown in Figure 5A indicate that the cytostatic effects of endogenous NO production do not result in an increase in DNA fragmentation in any of the cell lines. An analyses of the cell cycle profile by flow cytometry (Figure 5B) also indicated that IFN $\gamma$  treatment did not induce apoptosis in CL-2 and CL19 cells; however, CL-2 and CL-19 cells accumulated at G<sub>2</sub>/M (4N DNA content). Therefore, growth inhibition of these cell-lines by IFN $\gamma$  may be a result from the cell-cycle arresting at

G<sub>2</sub>/M. No changes in cell cycle were observed in response to IFN $\gamma$  in Renca cells.

### Effect of IFN $\gamma$ treatment on arginase activity

The differential expression of ARG2 previously observed (23) led us to test whether IFN $\gamma$  treatment affected ARG activity and L-arginine concentrations. Cytoplasmic extracts of untreated and treated cells with IFN $\gamma$  were isolated at 24, 48, and 72 h and tested for ARG activity. The three cell lines express similar basal ARG activity at 24 h. After 48 h, there was a slight increase in CL-2 and Renca cells, while a marked increase in CL-19 cells was observed, as illustrated in Figure 6. Immunoblot analysis for ARG2 protein confirmed these results (data not shown). The increase in basal ARG activity within the culture times

is observed only in the absence of media changes during the time course of the experiments. Changes were not observed in both untreated and treated cells with IFN $\gamma$ . Further, treatment with IFN $\gamma$  produced a significant decrease in ARG activity in CL-2 ( $p=0.048$ ) and CL-19 ( $p=0.003$ ) cells after 48 h, an effect that was only observed in Renca cells ( $p=0.031$ ) after 72 h in culture (Figure 6). Collectively, these data indicate that IFN $\gamma$  interferes with ARG activity in the three cell lines, perhaps by inhibiting transcription of the ARG2 gene.



**Figure 4. Nitrite (NO<sub>2</sub><sup>-</sup>) production measured in mRCC cells after treatment with IFN $\gamma$  and IFN $\gamma$  plus LNMMA.** Average nitrite concentration between untreated and treated cells with IFN $\gamma$  over time in (A) CL-2 (\*  $p=0.003$ ) and (B) CL-19 (\*\*  $p=0.037$ ) at 48 and 72 h respectively. Significant reduction of nitrite production (\*\*\*) ( $p<0.0001$ ) in CL-2 and CL-19 cells after the addition of iNOS inhibitor LNMMA. (C) Nitrite values in Renca cells after IFN $\gamma$  treatment. Results are the mean  $\pm$  SE of triplicates of five different separate experiments.

### Effect of IFN $\gamma$ in mRCC cell lines on L-arginine concentration and its effect on the production of NO and citrulline

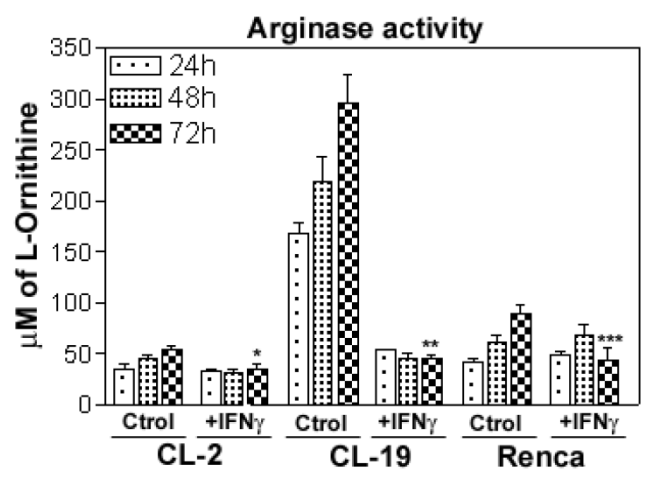
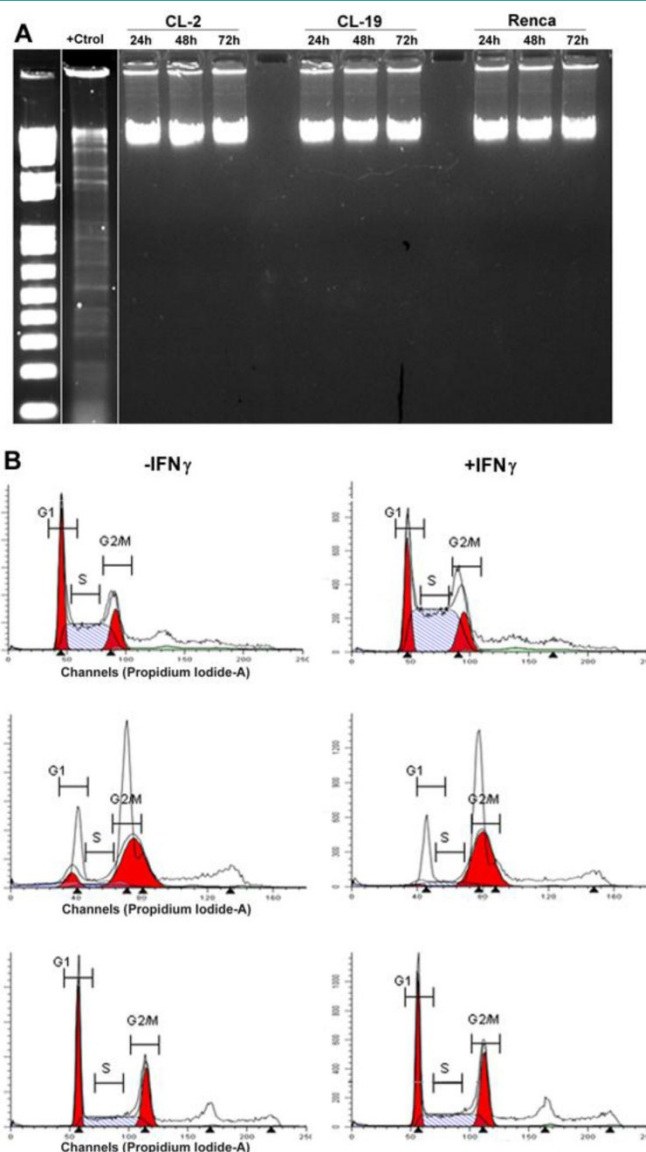
Since L-arginine is the common substrate for iNOS and ARG, we wanted to explore the effect of IFN $\gamma$  treatment on L-arginine consumption by HPLC. The standard concentration of L-arginine in the cell culture media was 1,140  $\mu$ M for all experiments. As seen in Figure 6A, in the first 24 h, the depletion of L-arginine was similar in the three cell lines. The CL-19 cell line, which expressed the highest levels of ARG activity, significantly depleted the media of L-arginine concentrations by 6-fold (450  $\mu$ M) at 24 h, 7-fold (296  $\mu$ M) at 48h, and 9-fold (110  $\mu$ M) at 72 h; however, no major changes in L-arginine content were observed in CL-2 and Renca cells at 48 and 72 h in culture. The addition of IFN $\gamma$  to the cultures significantly ( $p=0.0002$ ) decreased the rate of depletion of L-arginine in CL-19 at 72h when compared to untreated cells at the same time point (Figure 7A). The levels of L-arginine in CL-2 and Renca cells remained unchanged after the treatment. Concurrent with the depletion of L-arginine in the three cell lines, there was an increase in L-ornithine production that was abrogated ( $p=0.018$ ) after 72 h of IFN $\gamma$  treatment in CL-19 cells when compared to CL-2 and Renca as illustrated in Figure 7B. As iNOS catalyzes the conversion of L-arginine to equimolar quantities of NO and citrulline, the samples were analyzed for citrulline after treatment with IFN $\gamma$ . Citrulline concentrations increased significantly in a time-dependent manner in CL-2 ( $p=0.0001$ ) and CL-19 ( $p=0.0013$ ) at 48 h, whereas no increase was observed in Renca cells (Figure 7C). The data obtained for citrulline were nearly identical to the values obtained for nitrites seen in Figure 4, after IFN $\gamma$  treatment. Together, these results suggest that IFN $\gamma$  shifts the L-arginine metabolic pathway towards NO and citrulline production, consequently inhibiting RCC cell proliferation.

### Cytokine Production in mRCC cell lines

In order to gain insights into the biology of mRCC cell lines and to determine their ability to produce cytokines, we tested for IL-2, IL-4, IL-5, IL-10, IL-12 (p70), GM-CSF, IFN $\gamma$ , TNF $\alpha$ , and VEGF concentrations in the culture supernatants. Figure 8 shows the cytokine expression in the three cell lines at 48 h. We choose 48 h since the maximum production of these cytokines was observed at this time point. Moderate concentrations of IL-1 $\beta$  and IL-6 and high concentrations of VEGF and TGF $\beta$  were observed in all three cell lines. It appears that IFN $\gamma$  treatment had a significant effect in the production of IL-1 $\beta$  and IL-6

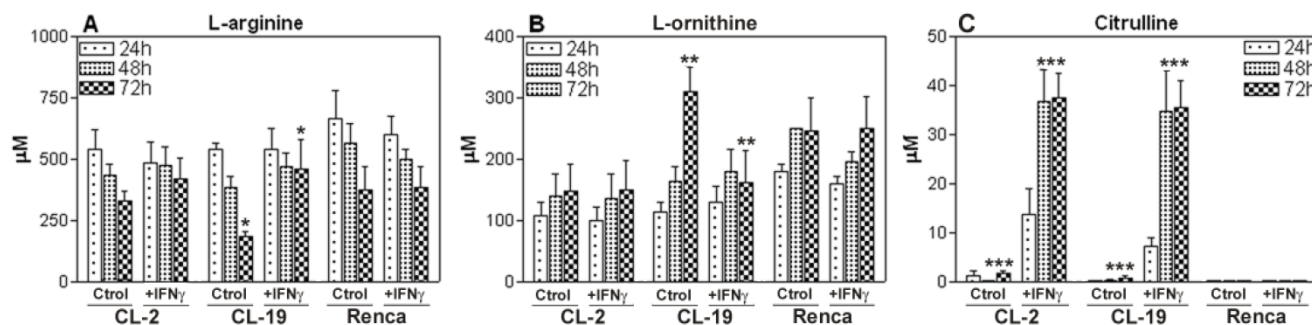
( $p= 0.001$  and  $p= 0.028$ ) in CL-2 and IL-1 $\beta$  ( $p= 0.09$ ) in CL-19 cells (Figure 8). This effect was not observed in Renca cells. Although, the amount of VEGF and TGF $\beta$  was considerably higher in all three cell lines at basal level, treatment with IFN $\gamma$  had no significant effect on the concentrations of these cytokines.

**Figure 5. Effect of IFN $\gamma$  treated cells on apoptosis and cell cycle.** (A) Cells were plated for 1 day and then cultured in media containing IFN $\gamma$ . DNA was extracted and subjected to DNA fragmentation ladder analysis. Data are representative of three independent experiments with similar results. (B) Analysis of cell cycle progression in untreated and treated mRCC cell lines with IFN $\gamma$ . After 24, 48, and 72 h cells were analyzed for DNA content by flowcytometry. A representative experiment performed at 48 h indicate the distribution of the cells in G1, S, and G2/M phases of the cell cycle.

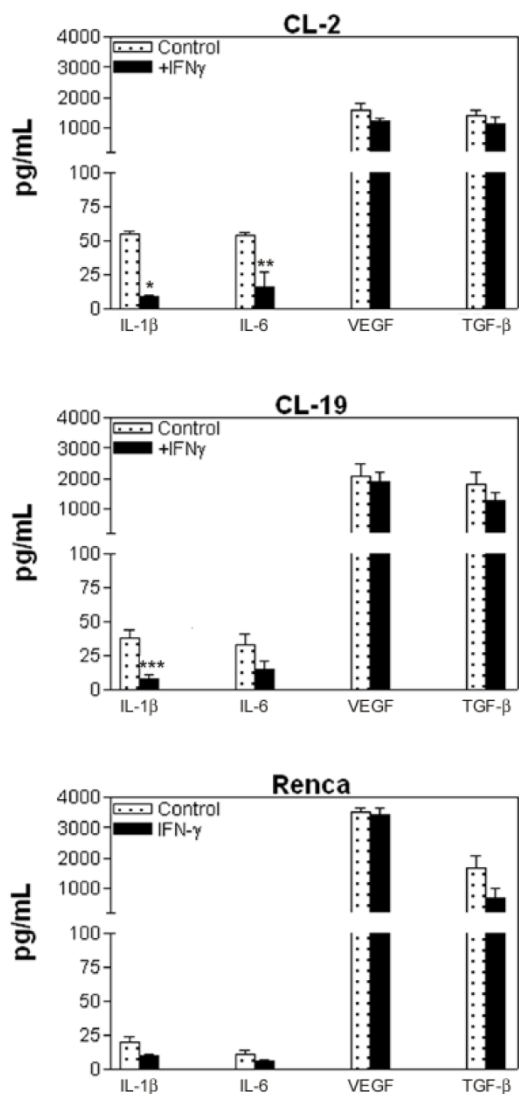


**Figure 6. The effects of IFN $\gamma$  on the induction of arginase activity in the mRCC cell lines.** Cells were treated with 10 U/ml of IFN $\gamma$  for 24, 48, and 72 h and tested for arginase activity (conversion of L-arginine to L-ornithine by arginase). As compared to untreated cells (Ctrl) significant reduction of ARG activity is observed: CL-2 (\*  $p= 0.02$ ) CL-19 (\*\*  $p= 0.0008$ ) and Renca (\*\* $p= 0.03$ ) at 72 h. The data represent the mean  $\pm$  SE of triplicate determinations from five separate experiments.





**Figure 7. L-arginine, L-ornithine, and citrulline levels.** Culture supernatants of CL-2, CL-19 and Renca cells untreated and treated with IFN $\gamma$  were collected at 24, 48, and 72 h and analyzed by HPLC after deproteinization with methanol and derivatization with OPA. Standards for L-arginine, L-ornithine, and citrulline in methanol were run with each experiment. Asterisks show significant differences between untreated and treated cells. **(A)** CL-19 L-arginine (\*  $p=0.0002$ ), **(B)** CL-19 L-ornithine (\*\*  $p=0.0183$ ), and **(C)** CL-2 and CL-19 citrulline (\*\*\*)  $p=0.0001$ ). The results are expressed as mean  $\pm$  SE of duplicate determinations from four independent experiments.



**Figure 8. Cytokine profile in supernatants of mRCC cell lines.** The cell lines were seeded in 6 well plates and treated with IFN $\gamma$  (10U/ml) for 24, 48, and 72 h. Supernatants were collected and assayed using a Th1/Th2 panel Bio-Plex cytokine assay. IL-1 $\beta$ , IL-6, VEGF, and TGF $\beta$  levels are shown. Treatment with IFN $\gamma$  has a significant inhibitory effect in CL-2 cells production of IL-1 $\beta$  and IL-6 in CL-2 (\*  $p<0.001$ ; \*\*  $p=0.002$ ) and IL-1 $\beta$  in CL-19 (\*  $p=0.009$ ). Data are representative of experiments at 48h of at least 3 independent experiments.

## DISCUSSION

In this study, we reported the following: 1) a new mechanism to define the cytostatic role of NO in the inhibition of RCC cell proliferation; 2) the effect of IFN $\gamma$  in modulating L-arginine metabolism and cytokine production; 3) the resistance of certain RCC cell lines (Renca) to IFN $\gamma$  treatment; and, 4) new insights into the mechanisms by which RCC tumors may locally and systemically shut down an important effector arm of the immune response. IFNs have been widely used in preclinical models and in immunotherapy trials for RCC (32;33). The activity of monotherapy with IFN $\alpha$  in metastatic RCC has been extensively evaluated (34;35) with disappointing and controversial results. However, trials using a combination of IL-2 and IFN $\alpha$  indicated a clinically useful anti-tumor activity with a 15% overall response (36;37). In contrast, therapy with the single agent IFN $\gamma$  has shown discouraging results in patients with RCC. Although preliminary studies suggested the benefits of IFN $\gamma$  treatment (38), only a 4.4% overall response has been seen in phase III studies indicating that IFN $\gamma$  alone has no major impact on RCC treatment (39).

These results are striking since many IFN $\gamma$ -mediated properties (e.g. anti-proliferative, apoptotic, and anti-angiogenic activity) have been attributed to this cytokine (40;41).

Our study was aimed to determine the mechanism(s) by which IFN $\gamma$  is capable of inducing growth inhibition of murine RCC cell lines. Previously, we reported that murine CL-2, CL-19, and Renca cell lines have different levels of ARG2 expression that appear to be associated with the enhancement of cell proliferation (23). The results indicate a need for the tumor cells to use ARG2 to metabolize L-arginine for the synthesis of polyamines to promote cell growth. Conversely, NO is a free radical that, at low concentrations, acts as single signal transducer whereas at higher concentrations can act as a cytotoxic/cytostatic defensive mechanism against pathogens and perhaps tumors (42). In our study, we found that RCC cell lines in the logarithmic phase of proliferation expressed low iNOS protein levels and low concentrations of NO as measured by the production of nitrites (15 -20  $\mu$ M), whereas ARG2 activity was highly present in the 3 cell lines. These results indicate that low iNOS and high ARG2 activity favor the growth of the cell lines and are supported by previous studies showing that high expression of ARG can inhibit iNOS activity and NO production (43-45). We believe this could be a mechanism used by tumor cells to survive by directly avoiding the cytostatic effects of NO.

Since IFN $\gamma$  is a potent inducer of iNOS, we wanted to determine *in vitro* the mechanism(s) by which this cytokine is capable of inhibiting RCC cell proliferation. We successfully demonstrated that iNOS induction within the tumor cells in response to IFN $\gamma$  caused a robust and sustained accumulation of endogenous NO that resulted in an 80-85% growth inhibition of CL-2 and CL-19 cell lines (Figure 3). This effect was abrogated by the addition of the iNOS inhibitor, L-NMMA, showing that the reduction in cell growth was mediated by NO. It is possible that the NO-mediated cytostatic effects observed in our *in vitro* model could produce a different outcome in an *in vivo* model due to the presence of NO-producing cells infiltrating the tumor microenvironment. Here, we found that the cytostatic effect of NO in RCC cell lines was not due to apoptosis as demonstrated by others (46;47), but rather by a cell cycle arrest at the G<sub>2</sub>/M phase. This possibly could be mediated by the transcriptional modulation of cyclins and/or cyclin-dependent kinases as seen in breast cancer cell lines and T cells (48;49). Since the growth-inhibitory activity of IFN $\gamma$  appears to be accompanied by the inhibition of ARG activity in CL-2 and CL-19 cells

(Figure 5), we are suggesting that this inhibition could be partially responsible for the growth-inhibitory effects of IFN $\gamma$  in addition to the major cytostatic effects of NO. The importance of ARG and/or NO in the growth-inhibition of RCC cells needs to be explored further by possibly using ARG and iNOS-KO mice. Altogether, our data suggest that ARG2 and iNOS can be modulated in a coordinated fashion by IFN $\gamma$  to counterbalance tumor growth and tumor inhibition. Lately, NO has emerged as a potential anti-proliferative agent capable of overcoming tumor cell resistance to conventional therapeutic agents. Despite the relative beneficial effect of IFN $\gamma$ -NO induction which inhibits RCC proliferation, a substantial percentage (15%) of the cells failed to respond to IFN $\gamma$  treatment. For the first time, our results show that a certain subpopulation of RCC cells develop a mechanism of resistance to specific cytostatic stimuli that could largely explain the commonly observed lack of response to IFN $\gamma$  in clinical trials. Mechanisms of resistance have been associated with IFN $\alpha$  defective Jak/Stat signaling pathways (50-52) that could be similar with IFN $\gamma$ .

Interestingly, in our study, we showed that Renca cells which, upon stimulation with IFN $\gamma$  express iNOS mRNA, did not express iNOS protein or NO production (Figure 2). Different mechanisms have been postulated to explain the lack of iNOS expression, but a novel finding has shown that loss of iNOS protein expression is exerted by translational inhibition specifically mediated by miR-146a (50). The goal of this study was not to understand the control of iNOS expression at the translational level, but to highlight that different RCC cell types can vary in their sensitivity to IFN $\gamma$  and, therefore, to NO. These findings will have a high translational relevance because permitting us to identify RCC cells better and to determine their sensitivity to IFN treatment. It is possible that human RCC could present with similar characteristics to those seen in Renca cells. Therefore, these cells (or patients) will not benefit from IFN $\gamma$  treatment not because IFN $\gamma$  does not work but rather because these cells found a way to become resistant to this cytokine as a mechanism of survival. Therefore, in patients, the identification of resistance to INF treatment could be extremely important for a more personalized and effective treatment of RCC.

Production of either stimulatory or inhibitory cytokines by infiltrating or neoplastic cells may have more of an impact on the development of an effective anti-tumor response than in the promotion of tumor growth. When we tested cytokine production before IFN $\gamma$  treatment, IL-1 $\beta$ , IL-6, TGF $\beta$ , and VEGF were the

most predominant cytokines in all 3 cell lines. Our results showed a parallel effect of IL-1 $\beta$  and IL-6 reduction (CL-2 and CL-19) with the inhibitory effects of NO, an effect that could be associated with low ARG activity. Conversely, IFN $\gamma$  did not have an effect on the production of VEGF and TGF $\beta$ , indicating that the inhibition of these cytokines can be mediated by other signaling pathways and that their presence can contribute to the maintenance of tumor growth and angiogenesis. A combination therapy of IFN $\gamma$  with other drugs (e.g. sunitinib) which targets VEGF receptors could advance the treatment of RCC.

In summary, we provided new evidence that an improved understanding of IFN $\gamma$  biology in the inhibition of tumor cell proliferation will provide new insights regarding the use of this cytokine, not only for the treatment of RCC, but for the treatment of other tumors where the use of interferons is indicated. Although the development of agents targeting VEGF and mTOR pathways has provided chemotherapeutic agents that are more active and less potentially toxic than interferons, regardless of the specific target, the end point of these therapies relies upon the induction of Th1 responses (mainly IFN $\gamma$  expression). Our results indicate that IFN $\gamma$  can play a key role in modulating L-arginine metabolism by inhibiting ARG activity and by enhancing the endogenous production of NO to inhibit the proliferation of RCC cells. Since numerous conflicting results about the role of iNOS and NO in cancer have been reported (53), understanding the multifaceted role of NO in cancer is imperative. The biochemistry, type of tumor, and the spatial and temporal effects of its production need to be more carefully studied in association with IFN $\gamma$ . Our findings can have multiple implications in RCC treatment not only by eliminating tumor cells and inhibiting angiogenesis, but more importantly by identifying the sets of patients with RCC that will benefit from IFN $\gamma$  treatment.

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## Conflict of Interest

All authors have declared that no conflicts of interest exist and agree with the contents of the manu-

script for publication.

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