**Supplemental Information**

**Arginase Producing Suppressor Myeloid Cells in Renal Cell Carcinoma Patients: A Mechanism of Tumor Evasion**
*Zea et al.*

## 1. Detailed Materials and Methods

***Samples:*** *P*re-treatment peripheral blood mononuclear cells from 123 metastatic RCC patients who participated in a Cytokine Working Group (CWG) Phase III trial (Mcdermott et al *JCO in press*) and 33 normal controls with similar age and gender distributionswere studied for arginase activity and CD3ζ chain expression. Additional samples from 15 RCC patients and 10 normal controls were used for cell separation studies at a later time.

***Flow cytometry:*** Surface markers for T cells, NK cells, monocytes and dendritic cells including CD3, CD4, CD8, CD15, CD16,CD56, CD14, CD86, CD83, MHC class I and II, CD11a, CD80 and intracellular CD3ζ were tested. Results were expressed both as percent positive cells and mean fluorescence intensity (MFI).

***Arginase Activity:*** PBMC and purified subpopulations were lysed and cytoplasmic extracts were tested for arginase expression by western blot and arginase activity by the conversion of L-arginine to L-Ornithine (nanomols of Ornithine/million cells/hour) as described elsewhere (2). Briefly, 25 μl (50 μg) of lysate were added to 25 μl of 10mM $MnCl_2$ at 55$^{o}$C for 20 min to activate arginase. Then 150 μl of 100mM carbonate buffer (Sigma) and 50 μl of 100 μM L-arginine were added at 37 $^{o}$C for 20 min. The reaction

was stopped with 750 μl of glacial acetic acid and 250 μl of ninhydrin was added and heated to 95$^{o}$C for 1 h. Samples were cooled at room temperature and absorbance read at 515 nm (Benchmark spectrophotometer -BioRad, Hercules, CA).

***RT-PCR:*** RNA from 2x10$^{6}$ PBMC or purified cell subsets were extracted using TRIzol (Invitrogen, Carlsbad, CA), treated with DNAse I and reverse transcribed using Superscript II. PCR amplification was developed using the following primers:

*Ase I:*

sense 5'-CAAGGTGGCAGAAGTCAAGA-3';

anti-sense:5'-GTCCAGTCCGTCACCATCAA-3'.

*Indoleamine 2,3-dioxygenase (IDO):*

Sense:5'-GTTCCTTACTGCCAACTCTC-3'

Anti-sense:5'ACACCAGACCGTCTGATAG-3';

*β-actin:*

sense 5'-CATGGGTCAGAAGGATTCCT-3'

anti-sense,5'-AGCTCGTAGCTCTTCCTCCAG-3'

The fragment sizes were: Ase I, 466 base pairs (bp), IDO:413 bp and β-actin, 661 bp. PCR products were visualized in ethidium bromide agarose gel.

*Western Blots:* Thirty (30) μg of cell lysates were electrophoresed in 10% Tris-glycine gels (Invitrogen Life Technologies, Carlsbad, CA), transferred to PVDF membranes (Invitrogen) and immunobloted with anti-human-Ase I , Ase II (Santa Cruz Biotech., Santa Cruz, CA) and GAPDH (RDI, Flanders, NJ), and detected by horseradish

peroxidase conjugated antibodies and ECL (Amersham Biosciences, Little Chalfont, UK). Gels were autoradiographed on X-OMAT AR films (Eastman Kodak, Rochester, NY).

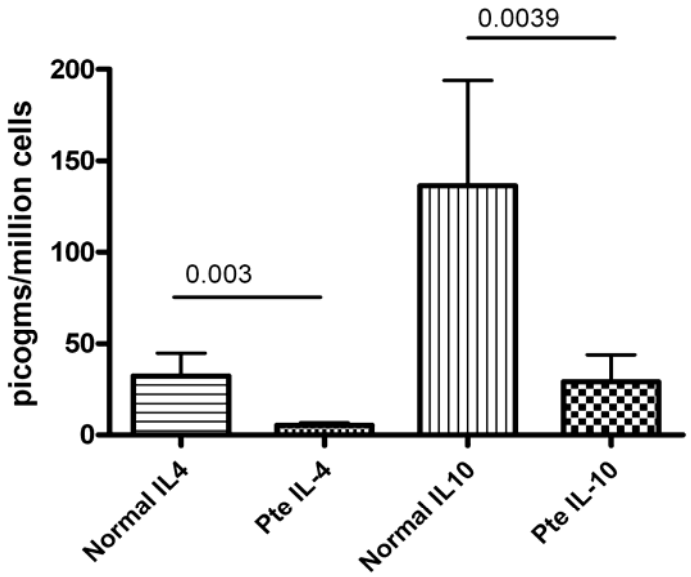
*L-arginine and L-ornithine serum levels:* High performance liquid chromatography coupled to electrochemical detection (HPLC-ECD) was performed using an ESA-CoulArray Model 540 (ESA Inc, Chelmsford,MA) with an 80 x 3.2 Column with 120A pore size (5). Sera was deproteinized and derivatized with 0.2M OPA/βME (o-phtaldialdehyde/β-mercaptoethanol). Fifty microliters were injected per sample. Standards of L-arginine in methanol were run with each experiment.

*Cytokine Measurement:* Supernatants from PBMC stimulated with anti-CD3 (30ng/ml) plus CD28 (100ng/ml) were collected at 48 and 72 hours and tested cytokine production using human Th1/Th2 panel Bio-Plex assay (BIO-RAD, Hercules, CA) following the manufacturer's instructions. Briefly, 96-well filter plates were pre-wetted and 50 μl of beads are added and washed twice with Bio-Plex buffer. Then, 50 μl of standards and samples are added, incubated for 30 minutes and washed 3 times. Detection antibodies (25 μl) were incubated for 30 minutes followed by 50 μl of streptavidin-PE for 10 minutes. The beads were re-suspended in 125 μl of buffer and read in a Bio-Plex array reader (BIO-RAD).

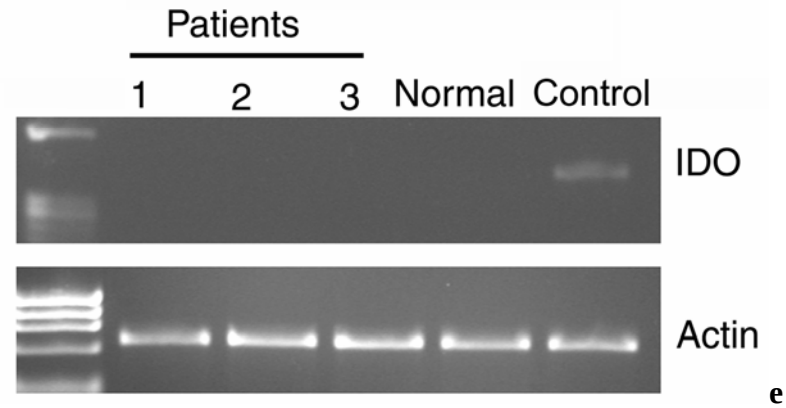
*Statistics:* Statistical analysis was done using GraphPad Prism 3.0 (Graph Pad software, San Diego, CA). Differences between the groups were determined by Wilcoxon's Rank

Sum test. Relationships between two variables were quantified by Spearman's Rank Correlation Coefficient .

**Supplemental Figure 1** . IL4 and IL10 production by PBMC from RCC patients and normal controls




**Supplemental Figure 2** . Absence of IDO (Indoleamine 2,3-dioxygenase) expression in CD11b+.CD14- suppressor cells as tested by RT-PCR.

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