ORIGINAL ARTICLE

Differential expression of efferocytosis and phagocytosis associated genes in tumor associated macrophages exposed to African American patient derived prostate cancer microenvironment

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ABSTRACT

Macrophages are the first line of defense in the cellular environment in response to any antigenic or foreign invasion. Since cancer cells express antigenic molecules and create a tumor microenvironment quite different from the normal cellular environment, macrophages will attack this cancer cells as foreign Invaders. However, the cancer cells adept their ability to suppress macrophage activity by secreting compounds/proteins through unknown mechanisms and train these macrophages to aid in tumorigenesis. These macrophages are commonly known as tumor associated macrophages (TAM). In this study, our goal was to find out key regulatory molecules involved in this conversion of cancer-fighting macrophages to cancer friendly macrophages. We used African American(AA) patient derived established human prostate cancer cells along with the human derived macrophages followed by Affymetrix cDNA microarray analysis. Microarray analysis of the PCa cell exposed macrophages revealed appreciable decrease in mRNA expression of several genes associated with phagocytosis process. Aberrant expression of several noncoding RNAs that control the expression of such phagocytosis associated molecules were also evident. Increased expression of oncogenic miR such as, miR-148, 615, 515, 130, 139 and markedly decreased expression of tumor suppressive miR's MiR-3130, let7c,101,103, 383 were noted. Further, TARGET SCAN analysis demonstrated these differential expression of non-coding RNA's causing down regulation of phagocytosis promoting genes elf5A, Meg3, Tubb5, Sparcl-1, Uch-1, Bsg(CD147), Ube2v, GULP, Stabilin 1 and Pamr1. There is an increase of RAP1GAP gene that causes concomitant decrease in the expression of tubulin genes that promote cytoskeletal assembly in forming phagosomes. In addition Ingenuity pathway analysis of the gene expression data also showed upregulation of antiphagocytic genes IL-10, CD16, IL-18 and MMP-9. Some core canonical pathways showing physiology of cellular signaling obtained by data analyzed by the Ingenuity software is confirmed a very complex mechanism still to be deciphered involved in the biology of TAM formation by which the rogue cancer cells tame their enemies, the macrophages and actually make them their helper cells to survive and propagate in the tumor microenvironment and thus prepare for epithelial mesenchymal transition for future metastasis and cancer stem cell formation and progression.

Key Words: Tumor associated macrophages, Tumor microenvironment, Core canonical pathways

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1. Introduction

Tumor-associated macrophages (TAMs) are a class of immune cells present in high numbers in the microenvironment of solid tumors. They are heavily involved in cancer-related inflammation. Macrophages are known to originate from bone marrow-derived blood monocytes (monocyte-derived macrophages) or yolk sac progenitors (tissue-resident macrophages), but the exact origin of TAMs in human tumors remains to be elucidated.[1] The composition of monocyte-derived macrophages and tissue-resident macrophages in the tumor microenvironment depends on the tumor type, stage, size, and location, thus it has been proposed that TAM identity and heterogeneity is the outcome of interactions between tumor-derived, tissue-specific, and developmental signals.[2] Although there is some debate, most evidence suggests that TAMs have a tumor-promoting phenotype. TAMs affect most aspects of tumor cell biology and drive pathological phenomena including tumor cell proliferation, tumor angiogenesis, invasion and metastasis, immunosuppression, and drug resistance.[3,4] However, it is not very clear how the cancer cells in the tumor micro environment (TME) influences the macrophages to switch sides and instead of fighting the cancer cells actually start helping them to grow and thrive. Henceforth in this study, we were interested to investigate the differential gene expression in the human macrophages in response to TME. Macrophages generally attack the pathogens or foreign invaders (including cancer cells which behave as foreign to the normal cellular homeostatic micro environment) by phagocytosis.[5–8] We, therefore, studied the effect of TME on the phagocytic index of these human macrophages by exposing them to human prostate cancer cells for 48 hours. We further did gene expression studies by the Agilent micro array analysis system and used the Ingenuity pathway analysis software program to determine the differential gene expression in these TME exposed human macrophages. We are describing the interesting findings on the effect of the TME on human macrophages in this manuscript.

2. Materials and Methods

2.1 Cell lines

The authenticated AA subject derived EOO6AA PCa cell line was obtained from ATCC (VA,USA) and cultured in RPMI 1640 medium supplemented with 10% FBS and Penn/Strep antibiotics, cell lines were maintained in a 5% Carbon Dioxide incubator. Human macrophage line (#CRL9856, ATCC) were cultured in ATCC recommended IMDM medium supplemented with 10% FBS, Penn/Strep antibiotics and maintained in a 5% Carbon Dioxide incubator. All cell lines were periodically checked for Mycoplasma contamination using a Mycoplasma detection kit (Sigma # MP-0025). All tissue culture media and reagents were purchased either from ATCC or Invitrogen.

2.2 Phagocytosis Assay

Human macrophage cells cultured by spinning the cell culture media of a confluent flask in a centrifuge for 10 minutes. The supernatant was discarded, and the pellet resuspended in fresh IMDM media. 500 μl of the resuspended pellet was distributed into wells on a 6 well cell culture plate and the contents of each well was brought up to 2 mL using fresh IMDM cell culture media. In the wells designated for co-culture, a 0.4 μm PET track-etched membrane cell culture insert was placed into the wells. The insert was then filled with leukemia cells prepared in the same method as the human macrophages using RPMI1640 as the media instead of the IMDM needed by the human macrophages. The cells were left in a 37°C incubator with 5% CO_2 for 48 hours. After the 48 hours, 5 μl of FITC labeled latex beads from the Cayman Chemical Phagocytosis Assay Kit (# 500290) was added to each well. The plate was then returned to the incubator for another 24 hours. To measure the amount of phagocytosis, the human macrophages from each well were removed, and spun in a centrifuge to collect the pellet. All cell culture media was removed, and the pellets were resuspended in PBS. Using a DeNovix QFX Fluorometer, the RFU of each sample was measured using the blue excitation channel with an excitation of approximately 470 nm with an emission range of 514-567 nm.

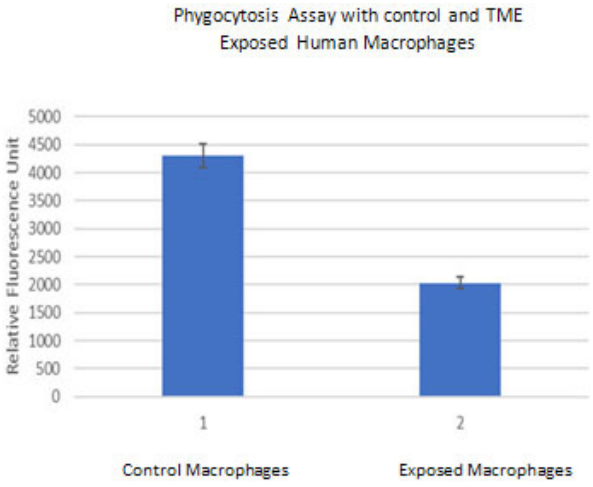

Figure 1. Differential phagocytic index in relative fluorescence unit (RFU) in the Y axis and TME exposed or unexposed Macrophages for 48 hours in X axis. There is a definite decrease in Pca TME exposed human macrophages phagocytic index

2.3 RNA sample preparation and Microarray analysis and gene expression profiling

Human prostate cancer cells were prepared to enable total cellular RNA isolation via Trizol (Invitrogen, CA, USA). The quantity of the RNA was determined with the Nano Drop ND1000 (ThermoFisher Scientific, CA, USA) and quality was verified with a Bio-analyzer 2100 (Agilent Technologies, CA, USA). Agilent Technologies Quick Amp Labeling Kit One Color was used to amplify 200 ng of total RNA into complementary RNA (cDNA) to use for oligo microarrays. The cDNA microarray analysis was done using the Whole Human Genome Oligonucleotide Microarray (G4112A, 41,000 genes; Agilent Technologies, CA, USA).

2.4 Microarray hybridization

To prepare the slides for microarray analysis they were hybridized using a buffer that included fluorescently labeled cDNA at 60°C for 17 h using HS Pro hybridization station. To wash the slides, 63x SSPE buffer that contained 0.005% N-lauryl sarcosine was used for the initial 1 min wash done at room temperature. After the first wash was finished, a second wash was performed using the 0.005% N-lauryl sarcosine containing 63x SSPE buffer that was pre heated to 37°C . The third wash was done using acetonitrile for 30 sec.

2.5 Image and data extraction

To measure the fluorescent signals from the hybridized microarrays, an Agilent and DNA microarray scanner with a resolution of 51 M and Agilent Feature Extraction Software (FES) was used. FES used the feature intensities and normalized ratios via linear LOWESS with background subtraction. The software rejected outliers and calculated statistical confidences (P-values). The only hybridization signals that were considered significant were those with P-values of less than 0.001. Genes were only considered relevant if they were differentially expressed in the three experimental replications.

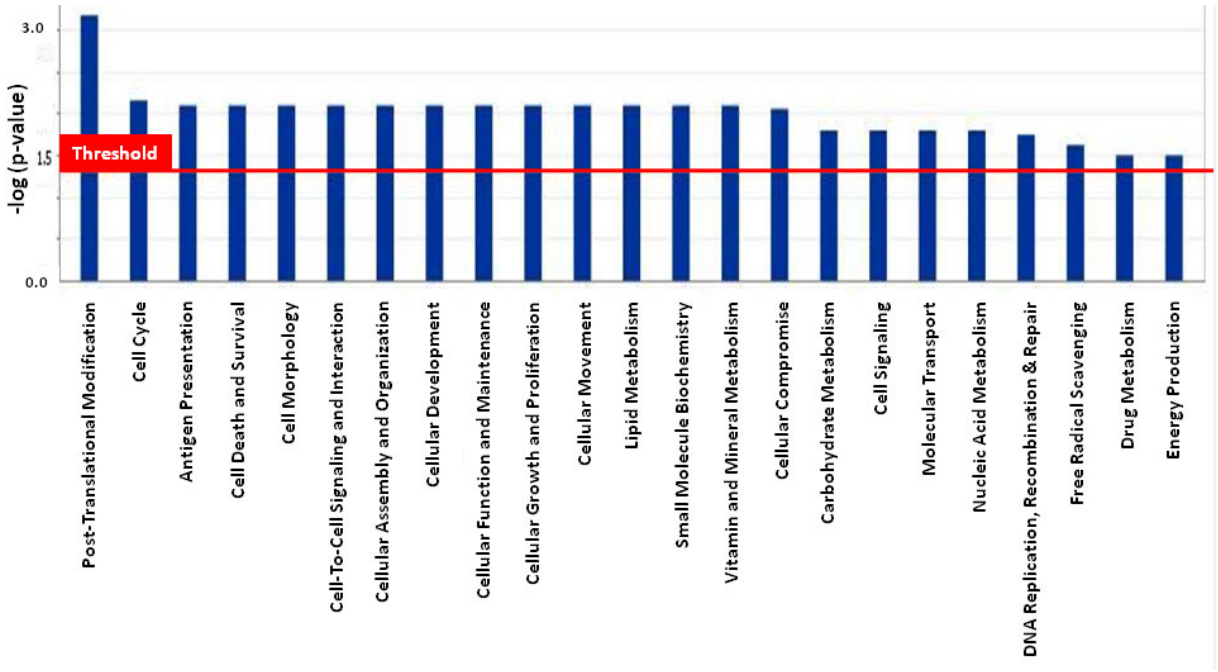

Figure 2. Ingenuity system analysis of top 500 upregulated and down regulated genes involved in different cellular pathways, these genes are differentially expressed in TME exposed human macrophages in comparison to unexposed controls.

2.6 Ingenuity pathway analysis (IPA) towards the identification of cellular processes and pathways

Data sets containing gene identifiers and corresponding expression values (fold change) were uploaded into Ingenuity Pathway Analysis software (Ingenuity® Systems, www.ingenuity.com). Each gene identifier was mapped to the corresponding gene object in the Ingenuity Pathways Knowledge Base. The information from the Ingenuity Pathways Knowledge Base (Genes Only) were used as references to consider direct and indirect relationships. Only the molecules and/or the relationships were included. An additional 661 gene transcripts were included in this analysis to improve the results. These 661 gene transcripts were found in the IPA knowledge base. Data sources from the ingenuity expert findings were utilized. To interpret the data in relation to biological processes, pathways, and networks the "Core Analysis"

function was used. Differentially expressed gene identifiers were defined as value parameters for analysis and identified the relationship between gene expression alterations and related changes in biofunctions under the subcategories of Molecular and Cellular Functions, Physiological System Development and Function, and Disease and Disorders. The genes found to have significant differential expression ($p <$ .05) were overlaid onto global molecular networks developed using information from the knowledge base. Networks were then algorithmically generated based on their connectivity. To name the networks, the most prevalent functional group(s) were used. To find the function of the specific genes in the networks, Canonical Pathway (CP) analysis was used.

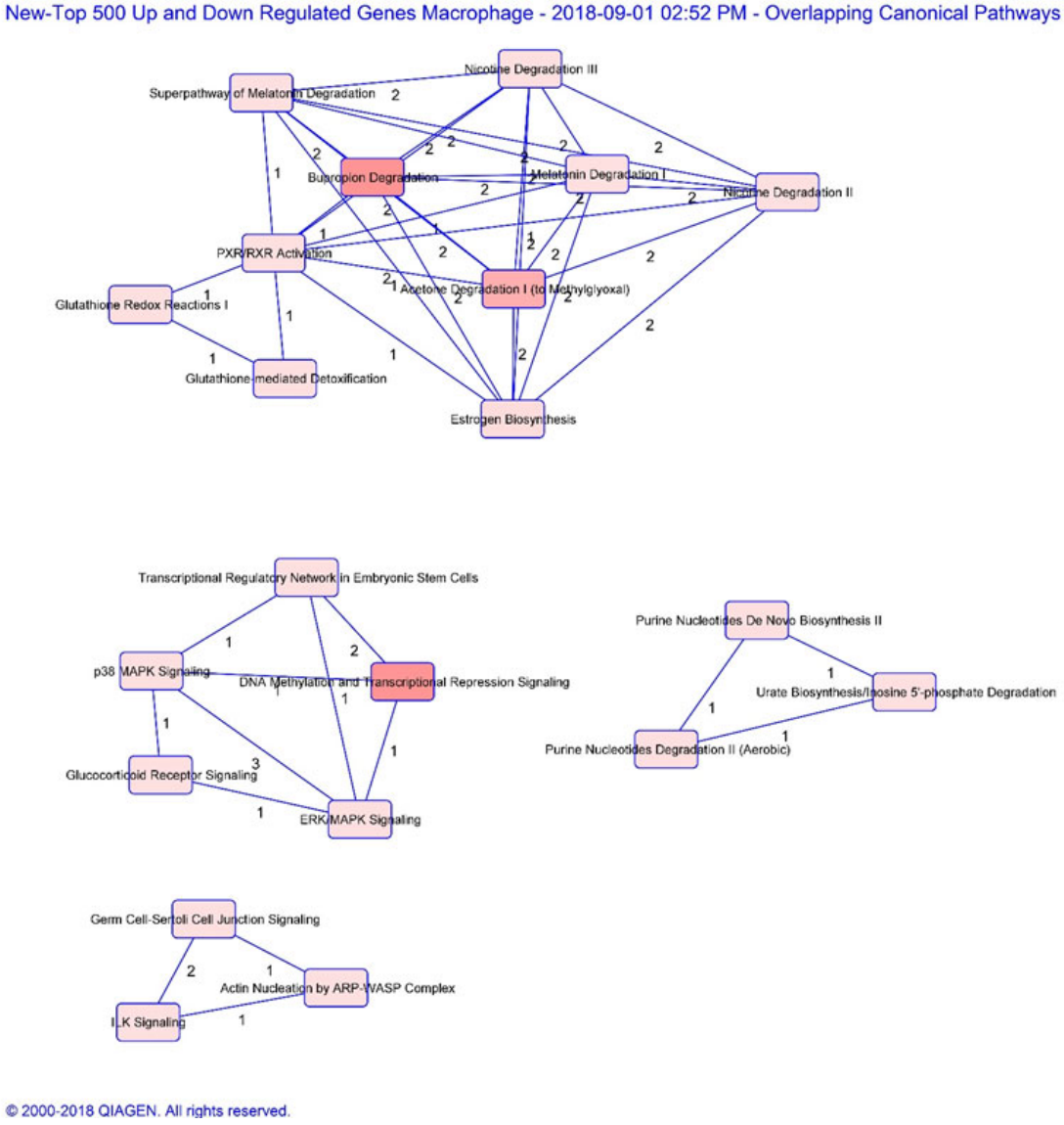

Figure 3. Core Canonical pathways as designed by the Ingenuity systems showing relationship between different cellular signaling events due to differential expression of genes in TME exposed human macrophages with respect to unexposed cells.

3. RESULTS

Our objective was to determine if there is differential gene expression in TME exposed macrophages in comparison to non exposed cells.

Our Phagocytosis results showed decreased phagocytic index in the tumor medium exposed macrophage cells as evidenced by the much lower relative fluorescence unit (RFU) recorded by the fluorometer in comparison to macrophages grown in absence of tumor medium (see Figure 1). Further analysis of the gene expression studies by PARTEK and Ingenuity

Systems software from total RNA isolated from these cells showed very interesting data validating decreased expression of several genes helping in the phagocytosis process and differential expression of several noncoding RNAs that control the expression of such phagocytic genes. The following gene expression of oncomiR's, miR 148, 615, 515, 130, 139, were increased and tumor suppressor miR's3130, let7c, 28, 101, 103, 383, 138 were decreased (see Figure 2). The TARGET SCAN Software results showed these differential expression of non coding RNA's causes down regulation of phagocytosis promoting genes elf5A, Meg3, Tubb5, Sparcl-1, Uch-1, Bsg(CD147), Ube2v, GULP, Stabilin 1 and Pamr1. There is an upregulation of RAP1GAP gene that causes downregulation of the tubulin genes that promote cytoskeletal assembly in forming phagosomes. Ingenuity Software analysis of the gene expression data also showed upregulation of antiphagocytic genes IL10, CD16. IL18 and MMP9. Some core canonical pathways showing physiology of cellular signaling were obtained by data analyzed by the IPA (see Figure 3). This confirmed a very complex mechanism still to be deciphered involved in the biology of TAM formation, by which the rogue cancer cells subdued the macrophages, and produce their own helper cell, to survive and propagate in the tumor microenvironment and thus prepare for epithelial mesenchymal transition for future metastasis and cancer stem cell formation and progression.

4. Discussion

TAMs are known to promote cancer growth survival and metastasis. M1 macrophages could fight the cancer cells by phagocytosis generating reactive nitrogen oxygen intermediates by inos activation. Il-12 like anti tumor cytokines.[9–11] However, by some unknown mechanisms, cancer cells in the tumor microenvironment switch monocytes and resident macrophages to M2 subtype which promotes Pro-tumor activity secreting IL1b, IL6, IL8, VEGF etc. It is known from the work of Dr. Carlos Crossi and other prominent cancer researchers in this field that IL4,IL13,IL10 and glucocorticoids secreted by cancer cells in TME helps in this switch to M2 macrophages which brings protumor activity; however what initial cellular changes in signaling, gene expression and core canonical pathways that are involved in conversion of M1 macrophages to TAM is still unknown.[12,13] Some recent studies[14] reported that immune checkpoint gene PD1 is upregulated in TAMs and blockage of PD1 by drugs and monoclonal antibodies resulted in increased phagocytic index and antitumor activity of TAMs in lung cancer model mice. Since we found down regulation of miR 28 and 138, two noncoding RNAs that work on PD1 mRNA, it could be worthwhile to further investigate the role of TME on PD1 expression on TAMs for therapeutic interventions. Interestingly, downregulation of phagocytosis promoting gene GULP and "EAT ME" signal identifier scavenger hunter receptor Stabilin gene down regulating micro RNA's were also upregulated, verifying our earlier results of the role of transmembrane protein GULP and its target receptor Stabilin in macrophage phagocytosis in TME.[15] Thus, we investigated further to decipher the early mechanisms involved in this switch by exposing human macrophages to tumor microenvironment and study the gene expression and phagocytic changes in comparison to unexposed controls. We found differential expression of several genes involved in phagocytic pathways. Notably, early expression of human cellular growth genes like VEGF, MMP9, angiogenesis and drug resistance genes like VEGF, A, C D and MMP2, invasion and metastasis related genes like VEGF, EGF, MMP9 and immune suppressors like IL10, TGF b and CCL22 were upregulated. Our preliminary findings could help in solving tumor associated macrophages formation and therapeutic interventions in the future, however more research needs to be done in elucidating the core canonical pathways involved and detailed studies of cellular mechanisms in formation of TAMs and novel drugs like Rhenium ligands in preventing TAM formation and rescue.[16]

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Conflicts of Interest Disclosure

The author declares no conflict of interest

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