

## Macrophage Polarization and the Effect of MicroRNA-155 Administered in Water-in-Oil-in-Water Multiple Emulsion Formulations

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### Abstract

The tumor microenvironment is composed of range of cells including macrophages that participate in sustaining tumor growth and invasion. Tumor-associated macrophages (TAM's) polarized to the M2 or pro-tumoral phenotype are one such population of cells highly expressed in the tumor microenvironment. The expression of microRNA's (miR's) is often found to be dysregulated in TAM's and hence exogenous delivery of miR provides a strategy for macrophage phenotypic modulation to attain improved anticancer activity. In this study, we assessed the use of multiple emulsions for the delivery of miR to achieve macrophage M1 repolarization. Water-in-oil-in-water (WOW) multiple emulsions (ME) were prepared using a two-step emulsification technique for encapsulation of miR-155 and cellular uptake, transfection efficiency, and repolarization capability of J774A.1 macrophages was assessed. The repolarized macrophages were co-cultured with SKOV3 ovarian cancer cells to assess the effect on macrophage morphology, motility and apoptosis of cancer cells. The ME showed enhanced uptake and expression of miR-155, which resulted in improved M1 polarization of J774A.1 cells. Co-culture studies with SKOV3 cells indicated an alteration in apoptotic profile. Holographic assessment of the co-cultured cells in real-time showed differences in motility and morphology of macrophages with miR-155 ME treated cells showing greater cellular interaction between the two phenotypes.

**Keywords:** Tumor-associated macrophages; Macrophage polarization; MicroRNA-155; Water-in-oil-in-water multiple emulsions

### Introduction

Cancer is one of the foremost public health concern around the world estimated to account for 13.1 million deaths out of the 22 million diagnosed cases each year by 2030 [1,2]. Even after great strides have been made in cancer research, development of a safe and successful universal clinical therapy has still not been achieved [3] For decades it was assumed that solid tumors are merely a collection of aneuploid cells that undergo uncontrolled proliferation leading to cancer. However, it has been realized that tumors are organ like assemblies, which are structurally complex, consisting of an eclectic mixture of multiple cellular entities belonging to different cell lineages that together form the tumor microenvironment (TME). The interplay of numerous cellular components with one other and extracellular matrix components in response to cues provided by signaling molecules and soluble factors in the vicinity provides functional support to the cancer cells [4-7].

TME is prominently populated by macrophages known as tumor-associated macrophages (TAM) in almost all types of malignant conditions with their number being as high as 65% in certain tumors [8,9]. Tissue-associated macrophages can be skewed to different phenotypic polarization states, such as M0, M1, M2, depending on the environmental cues provided and can exhibit a pleotropic mixture of both tumor-promoting and tumor-inhibitory actions [9] Clinical and experimental data has revealed that these TAM's recruited and residing in the TME are pre-dominantly polarized to M2 phenotype and are the major players that are involved in almost every stage of

cancer development starting from initiation to growth, immunosuppression, progression, angio and lymphangiogenesis and finally metastasis. On account of their central role in cancer etiology, TAMs have attracted a lot of attention as a target for anti-tumor therapy [10,11]. Out of the many strategies targeting these pro-tumoral macrophages, macrophage repolarization/reprogramming is a promising approach because it aims at re-education of TAMs to the anti-tumoral state, rather than causing their destruction [12,13].

RNA interference is an endogenous mechanism for regulating the expression of genes. This approach finds application in the induction of TAM repolarization either via translational repression of factors that promote M2 polarization or translational promotion of factors involved in M1 polarization. MicroRNA based interference is a suitable strategy since it can affect and regulate multiple factors as opposed to siRNA which can target just one gene. Among the various microRNA's that mediate macrophage polarization, MicroRNA-155 (miR-155) is important due to its effect on hematopoiesis, inflammation, cancer and immunity [14]. It has emerged as a key player in cancer and cancer immunity development having both pro and anti-tumoral effects depending on the cell type in which its expression is altered [15]. Delivery of microRNA faces many hurdles which need to be overcome before the immense potential of microRNA's can be harnessed. Several viral vector based miR systems have been developed to overcome these delivery challenges; however, due to their associated risks, alternative approaches have been explored. Synthetic non-viral delivery systems tend to have several advantages over their viral counterparts such as effective control over composition, low immunogenicity, easier manufacturing, scope for surface modifications and ease of analysis. However, such systems lack the high efficiency of delivery associated with viral vectors. Thus, there

is a need to develop systems that can be targeted and thus achieve better delivery efficiency [16]. We have previously investigated gene delivery using multiple emulsions systems for transfection of macrophages to attain enhanced intracellular delivery and gene transfection efficiency in J774A.1 cells [17]. The multiple emulsion system being a liquid formulation can be administered both orally and parenterally and can also serve as a multi-cargo system by utilizing the oil phase for concurrent delivery of a hydrophobic molecule. Additionally, the encapsulation of the DNA molecule inside the oil phase provides protection against degrading enzymes..

The aim of the current investigation was to prepare miR-155 encoding plasmid ME and assess the re-polarization of macrophages from M2 to M1 phenotype. This was followed by the functional assessment of this re-polarization on activity of SKOV3 ovarian adenocarcinoma cells.

## Materials and Methods

### *In vitro* M1 and M2 polarization of macrophages

J774A.1 murine macrophage cell line (ATCC<sup>®</sup> TIB-67<sup>™</sup>) was obtained from the American Type Culture Collection (ATCC; Manassas, VA) and grown using Dulbecco's modified eagle medium (DMEM; Mediatech Inc., Manassas, VA) supplemented with 10% fetal bovine serum (FBS; Fisher Scientific, Pittsburg, PA) and 1% Penicillin-Streptomycin-Amphotericin B antibiotic combination (Pen/Strep/Amphotericin B; Lonza Walkersville Inc., Walkersville, MD) in a T-25 flask at 37°C and 5% CO<sub>2</sub>. The cells were plated at a seeding density of 1 million cells per 5 ml and allowed to grow overnight before the polarization stimulus was added the following day. To promote M1 polarization, the cells were treated with 100 ng/ml lipopolysaccharide (LPS) and with IL-4 for M2 polarization for 6, 16, 24 and 36 hours. Following the stimulation, cells were harvested, RNA extracted and expression of M1 (TNF- $\alpha$ , IL-1 $\beta$ , iNOS) and M2 (Arg-1, IL-10) markers was assessed by qPCR analysis using the Roche LightCycler 480<sup>®</sup> instrument.

### Multiple emulsion-encapsulated mir-155 formulation, uptake and transfection in macrophages

Water-in-oil-in-water (W/O/W) multiple emulsion (ME) formulations that could encapsulate a water soluble payload were formulated using a two-step emulsification method as previously described [18,19]. The safflower oil used in making the oil-in-water primary emulsion was kindly provided by Jedwards International, Inc. (Braintree, MA). Span<sup>®</sup>80 and Pluronic<sup>®</sup> F-127 surfactants were purchased from Sigma-Aldrich Inc. (St Louis, MO) and BASF Corporation (Mount Olive, NJ) respectively. The ratio of Internal Aqueous: Oil: External aqueous phase was 1:2:3. Safflower oil-Span<sup>®</sup>80 Mixture (9:1) was used to make primary emulsion with RNase-free water by homogenization using homogenizer (Silverson's Model: L4RT-A; Silverson Machines, East Longmeadow, MA) at 10,000 rpm for 5 minutes. The primary emulsion thus formed was then re-emulsified with a 0.5% w/v solution of Pluronic<sup>®</sup> F127 in RNase free water by homogenization at 10,000 rpm for 15 minutes to create the blank multiple emulsions (BME) that does not encapsulate any payload. When formulating multiple emulsions encapsulating null plasmid vector DNA (NME) or miR-155 encoding plasmid DNA (MME), 500  $\mu$ l of the respective plasmids (7,000 ng/ $\mu$ l) in RNase free water were used to prepare a total of 3 ml of ME.

The uptake of miRNA encoding plasmid by J774A.1 macrophages, was studied by labelling 3.5 mg of plasmid DNA with 500  $\mu$ l of 1 mg/ml solution of Propidium Iodide (Sigma Aldrich, St. Louis, MO). The labeled plasmid DNA was then encapsulated in the internal aqueous phase of the ME. Lab-Tek 4 chamber slides (Thermo Scientific, Rochester, NY) were kept in UV light for 20-30 minutes. Thereafter approximately 200,000 cells were plated and allowed to adhere overnight before treatment with different conditions was started the next day. The cells were left untreated or treated with naked plasmid, BME or MME and incubated for 60, 120, 240 and 360 minutes respectively, with Hoechst 33342 (Life Technologies, Eugene, Oregon) being added to culture media in the recommended concentrations 15 min before the end of the incubation period. Following incubation, the cells were washed with sterile ice cold PBS and fixed with 3.6% w/v formaldehyde for 15 minutes. The chambers were then removed following the manufacturer instructions and a clean cover slip was then placed on the slide on which a drop of Immuno-Mount (Richard Allen Scientific, Kalamazoo, MI) was added. The slides were then visualized under a LSM 700 Confocal microscope (Carl Zeiss Microscopy) with Z-stack capabilities.

In order to ascertain the intracellular levels of miR-155 attained after various treatments. 200,000 cells were plated in 6 well plates and allowed to grow overnight. The following day the cells were either left untreated or treated with either naked plasmid (20  $\mu$ g) or Lipofectamine3000-Plasmid Complex or BME, NME or MME for 4 hours. Thereafter cells were washed thrice with PBS and then allowed to grow for 24, 48, 72, 96 and 120 hours. At each time point cells were harvested and the RNA was extracted using the Quick-RNA<sup>™</sup> MiniPrep Kit (Zymo Research Corp., Irvine, CA). The isolated RNA was analyzed quantitatively and qualitatively using Nano-Drop<sup>®</sup> 2000 (Thermo Scientific, Wilmington, DE). 100 ng of the RNA was reverse transcribed using the TaqMan<sup>®</sup> MicroRNA Reverse Transcription Kit (Life Technologies, Foster City, CA) and the recommended protocol. The cDNA obtained was then used in setting up qPCR reactions according to the recommended protocol using the TaqMan probes for mature mmu-miR155-3p (Life Technologies, Foster City, CA) using for snoRNA202 (Life Technologies, Foster City, CA) which was used as an endogenous control. The  $\Delta\Delta$ Ct Method was then used for determining the expression levels of miR155 and snoRNA202.

### miR-155 mediated macrophage repolarization

Following formulation development approximately 200,000 cells were plated in a T-25 flask and allowed to grow overnight. The following day the cells were left untreated or incubated with naked plasmid, miR155 expressing plasmid DNA complexed with Lipofectamine<sup>®</sup> 3000 (Life Technologies), blank multiple emulsions (BME), null plasmid vector-encapsulated multiple emulsion (NME) and miR-155 expressing plasmid encapsulated W/O/W multiple emulsion (MME) for 4 hours. Cells were washed twice with PBS and allowed to grow for 28 hours to enable the production of miR-155. Cells were then stimulated with 100 ng/ml IL-4 to induce their polarization to M2 phenotype, and incubated for an additional 16 hours. The effect of miRNA-155 on the expression of M1 and M2 phenotype specific marker genes was then assessed using qPCR. The time points chosen were 12 hours, 24 hours, and 48 hours post IL-4 stimulation.

### Macrophage polarization studies in co-culture with Skov3 ovarian tumor cells

Approximately 300,000 macrophages were plated in T-25 flasks and allowed to adhere overnight. These cells were left untreated or treated with LPS for 6 hours, IL-4 for 6 hours, naked plasmid and miR-155 plasmid encapsulated in ME's for 48 hours. Following the duration of macrophage stimulation, 50,000 cells were added to 50,000 SKOV3 cells in T-25 flask, and following 48 hours post co-culturing the M1 and M2 specific markers were assessed by qPCR. The expression of pro-apoptotic (APAF-1, Notch-1, Caspase-3) and anti-apoptotic markers (Bcl-2, Survivin) was assessed from SKOV3 cells.

### Label-free time lapse holographic imaging and analysis of treated macrophages co-cultured with skov3 ovarian tumor cells

Approximately 300,000 J774A.1 macrophage cells were plated in T-25 flasks and allowed to adhere overnight. These cells were left untreated or treated with LPS for 6 hours, IL-4 for 6 hours and miR-155 plasmid encapsulated in ME's for 48 hours. After aforementioned incubation with stimulants, J774A.1 macrophages were imaged for 6 hours at 5 min. intervals. For co-culturing, approximately 2,000 SKOV3 cells were plated in petri-dishes and allowed to adhere overnight. The stimulated macrophages after imaging as described above were added to the cancer cells and they were allowed to adhere to the plate for 6 hours. The interaction of the two-cell population was assessed using the HoloMonitor M4 (Phase Holographic Imaging, Lund, Sweden) and time-lapse videos were obtained. The macrophages were tracked over a period of 24 hours by imaging at 5min intervals and their motility patterns were assessed. 3D and 4D plots were developed to assess the interaction of these cells over a 24 hours period.

The HM4 HoloMonitor (Phase Holographic Imaging, Lund, Sweden) uses a low power 635 nm diode laser to obtain hologram images of unlabeled live cells. The instrument fits inside a standard CO2 controlled tissue culture incubator, and the associated computer is used to obtain time-lapse sequences of user selected image frequency and duration. Proprietary software is used to segment cells, quantify cell features and track cell locations. In addition, time lapse image series were exported into NIH freeware Image J, and processed as 4 dimensional image stacks (X and Y position) cell thickness (brightness) and time.

### Statistical data analysis

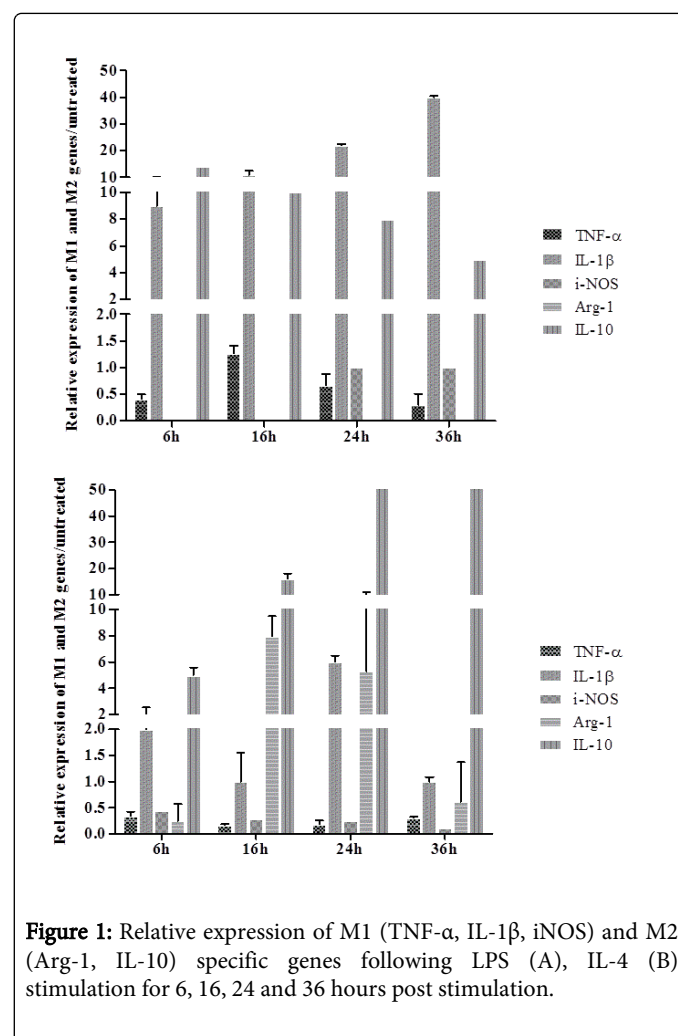
All the statistical analysis was performed using Prism<sup>®</sup> 5.0 software (Graph Pad Software Inc., San Diego, CA). Results were expressed as mean  $\pm$  SD of the at least three independent experiments. Data was analyzed by one way ANOVA followed by Bonferroni's post hoc analysis for multiple comparisons. Differences were considered statistically significant at  $p < 0.05$ .

## Results

### In Vitro polarization of macrophages

Figure 1A shows the relative expression of M1 and M2 specific genes post LPS and IL-4 stimulation for 6, 16, 24 and 36 hours. Following LPS stimulation, the J774A.1 cells showed an increase in M1 markers (TNF- $\alpha$ , IL-1 $\beta$ , iNOS) relative to untreated cells. Among these

markers, TNF- $\alpha$  and IL-1 $\beta$  increased gene expression within 6 hours post stimulation whereas iNOS levels increased within 24 hours post stimulation. There was steady increase in IL-1 $\beta$  expression over 36 hours post stimulation. With respect to M2 markers, no Arg-1 expression was detected with LPS stimulation. However, IL-10 demonstrated a steady decrease in expression over 36 hours of LPS stimulation. Figure 1B, shows the relative expression of the M1 and M2 specific markers post IL-4 stimulation. M1 markers, namely TNF- $\alpha$  and iNOS showed very low levels of expression with a very minimal change over the 36 hours of stimulation period. IL-1 $\beta$  on the other hand showed no clear trend in expression levels. Among the M2 markers, peak Arg-1 levels were observed over 16 to 24 hours with gradual decrease 36 hours post stimulation and IL-10 showed a gradual increase in expression up to 24 hours after which a plateau was reached.

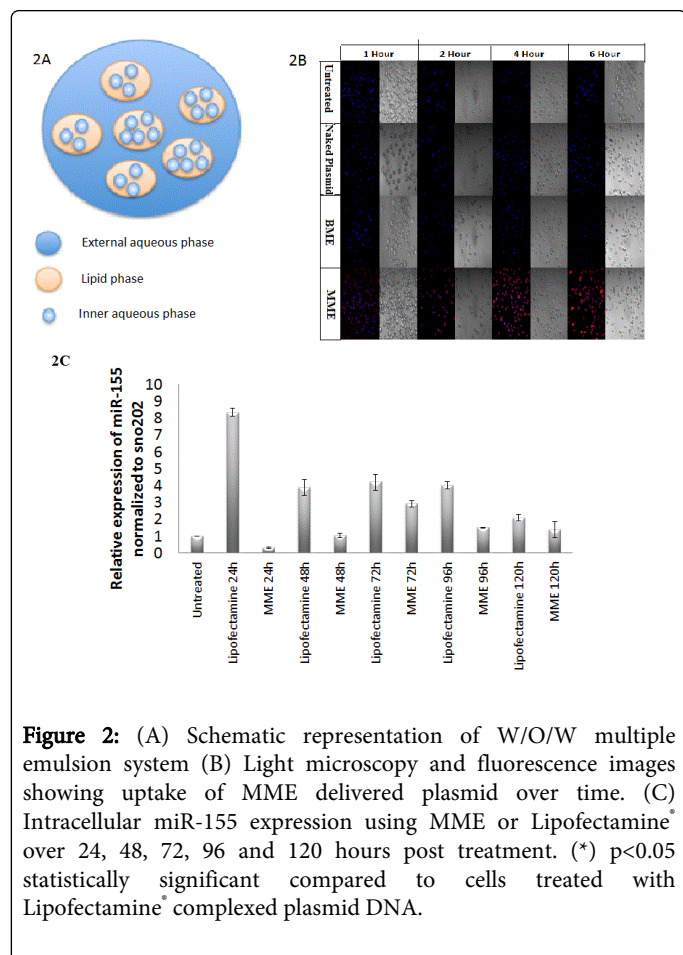


**Figure 1:** Relative expression of M1 (TNF- $\alpha$ , IL-1 $\beta$ , iNOS) and M2 (Arg-1, IL-10) specific genes following LPS (A), IL-4 (B) stimulation for 6, 16, 24 and 36 hours post stimulation.

### Multiple emulsion-encapsulated mir-155 uptake and transfection in macrophages

Figure 2B shows light microscopy bright field and fluorescence images of untreated J774A.1 cells or those incubated with naked plasmid, BME and MME for different time points, with Hoechst 33342 used to label the DNA (blue) It can be inferred that the multiple emulsion system encapsulating plasmid was rapidly taken up by J774A.1 macrophages starting as early as 1 hour after incubation. The

uptake increased as the time progressed and maximum uptake as evident from fluorescence signal was observed at the 6 hour post-incubation (Figure 2).

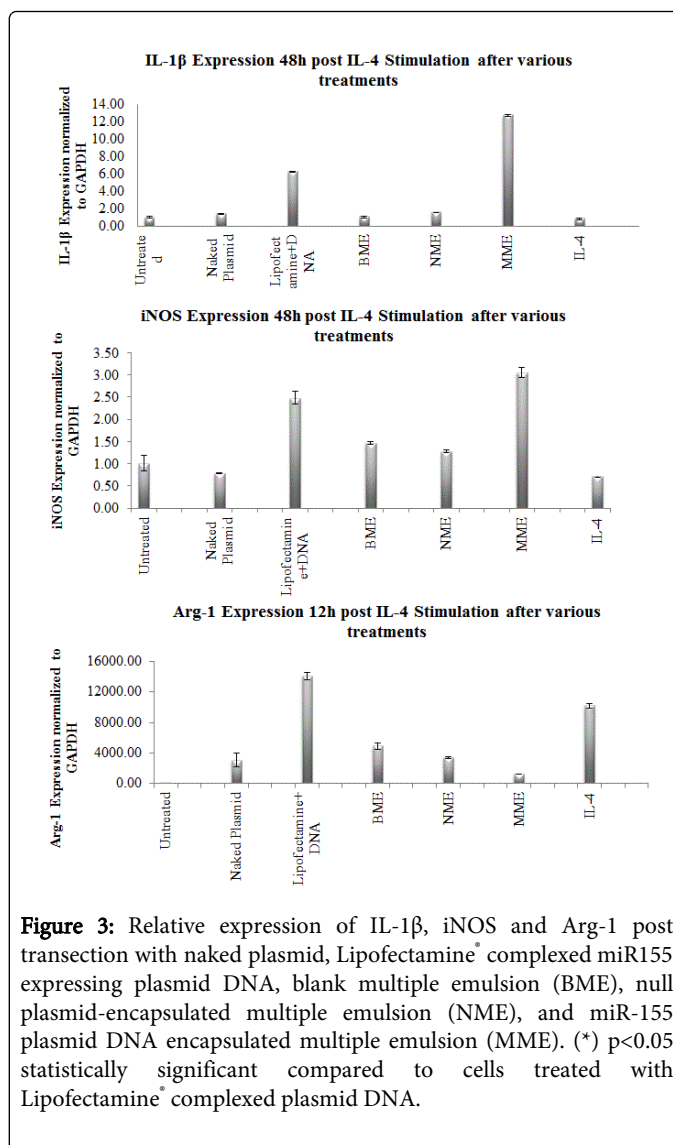


**Figure 2:** (A) Schematic representation of W/O/W multiple emulsion system (B) Light microscopy and fluorescence images showing uptake of MME delivered plasmid over time. (C) Intracellular miR-155 expression using MME or Lipofectamine<sup>®</sup> over 24, 48, 72, 96 and 120 hours post treatment. (\*)  $p < 0.05$  statistically significant compared to cells treated with Lipofectamine<sup>®</sup> complexed plasmid DNA.

Quantitative miR-155 expression studies indicated that with Lipofectamine<sup>®</sup> complexed plasmid DNA, peak miR-155 expression was obtained within 24 hours ( $p < 0.05$  compared to ME encapsulated miR-155) with a rapid decline in miR-155 expression within 48 hours. On the other hand, ME showed had a more delayed miR-155 expression occurring between 48-72 hours of incubation with a gradual decline over 120 hours.

### Re-polarization of macrophages with mir-155 encapsulated in multiple emulsions

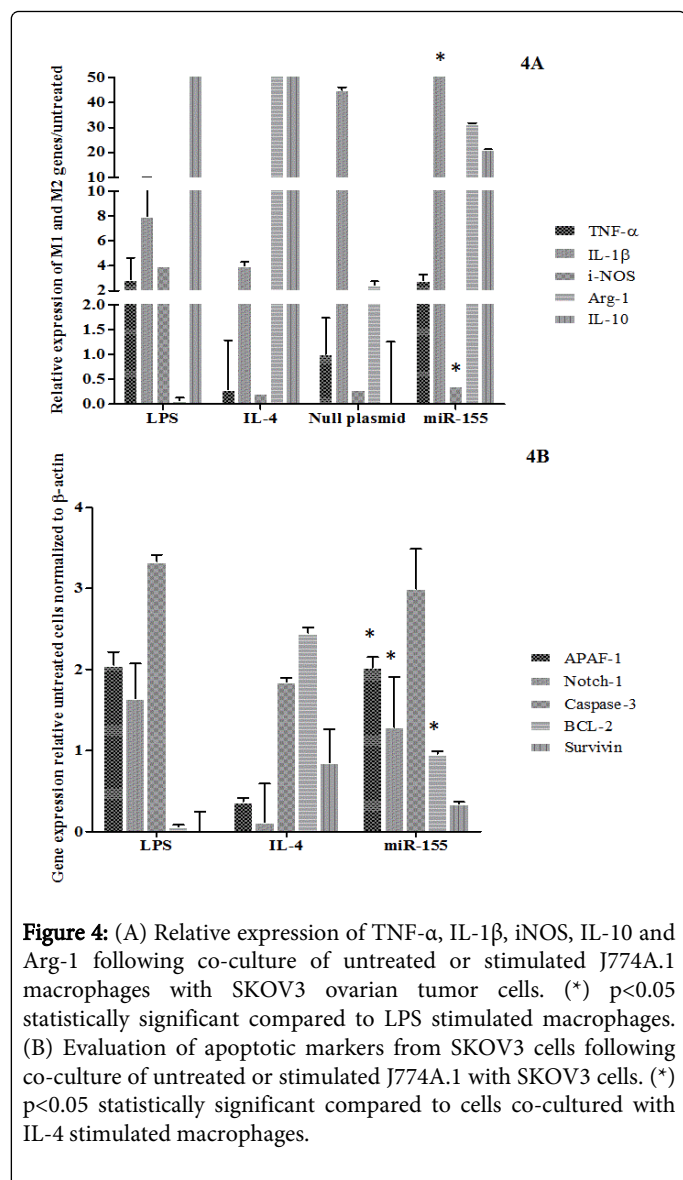
The results in Figure 3A show IL-1 expression 48 hours post IL-4 stimulation after various treatments. A 6-fold and 12-fold increase in IL-1 $\beta$  expression was observed following treatment with Lipofectamine<sup>®</sup> complexed miR-155 encoding plasmid DNA and ME encapsulated miR-155 plasmid DNA, respectively. Figure 3B, indicates the iNOS expression 48 hours post IL-4 treatment which shows ~ 3-fold increase with both Lipofectamine<sup>®</sup> complexed and multiple emulsion encapsulated miR-155 plasmid DNA. Figure 3C, shows the Arg-1 expression 12 hours post IL-4 stimulation. Approximately 10 fold decrease in Arg-1 level was observed relative to IL-4 stimulated macrophages. Thus, increased expression of M1 markers (IL-1 $\beta$  and iNOS) and decrease in M2 marker (Arg-1) was observed after treatment of M2 polarized macrophages with the plasmid.



**Figure 3:** Relative expression of IL-1 $\beta$ , iNOS and Arg-1 post transfection with naked plasmid, Lipofectamine<sup>®</sup> complexed miR155 expressing plasmid DNA, blank multiple emulsion (BME), null plasmid-encapsulated multiple emulsion (NME), and miR-155 plasmid DNA encapsulated multiple emulsion (MME). (\*)  $p < 0.05$  statistically significant compared to cells treated with Lipofectamine<sup>®</sup> complexed plasmid DNA.

### Macrophage polarization and apoptotic gene expression in co-culture with SKOV3 tumor cells

Figure 4A, depicts the effect of co-culturing SKOV3 cells with stimulated J774A.1 macrophages. The LPS stimulated macrophages indicated an increase in the M1 markers and no significant change in the Arg-1 levels compared to the untreated cells. In contrast the IL-4 stimulated cells showed a relative decrease in M1 and an increase in the levels of M2 markers. Once the behavior of LPS and IL-4 stimulated co-cultures was identified the effect of miR-155 encapsulated in multiple emulsion was assessed relative to the LPS and IL-4 stimulated macrophages. The miR-155 treated co-cultures showed an increase in TNF- $\alpha$  and IL-1 $\beta$  levels similar to LPS stimulated cells. However a consistent increase in iNOS was not observed. On the other hand the miR-155 transfected cells when co-cultured with cancer cells showed a decrease in Arg-1 and IL-10 levels (M2 specific markers).



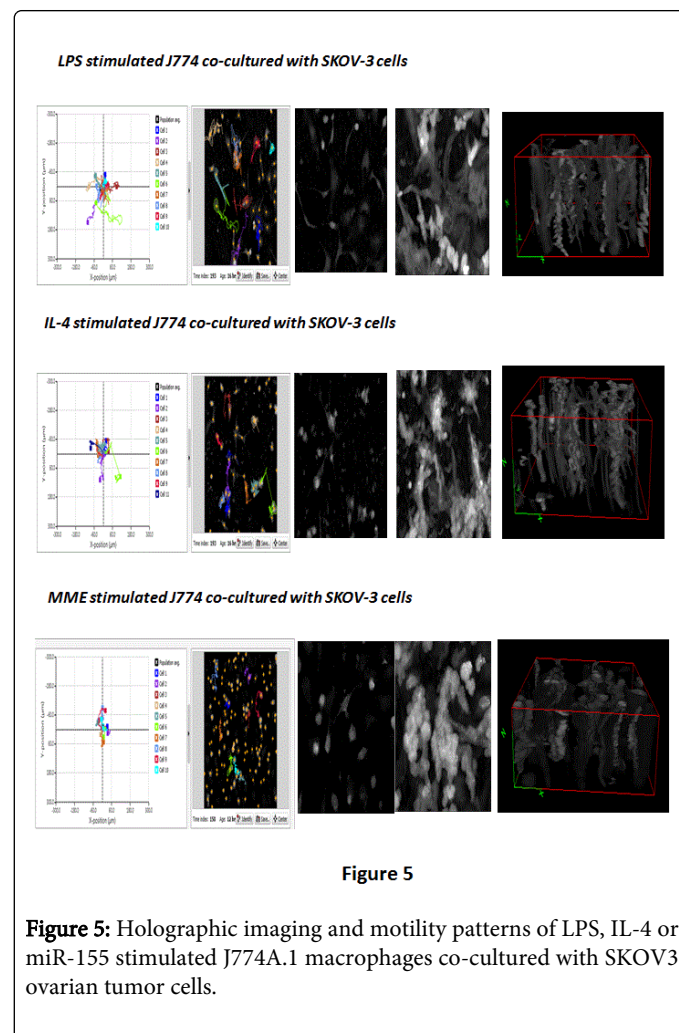
**Figure 4:** (A) Relative expression of TNF-α, IL-1β, iNOS, IL-10 and Arg-1 following co-culture of untreated or stimulated J774A.1 macrophages with SKOV3 ovarian tumor cells. (\*) p<0.05 statistically significant compared to LPS stimulated macrophages. (B) Evaluation of apoptotic markers from SKOV3 cells following co-culture of untreated or stimulated J774A.1 with SKOV3 cells. (\*) p<0.05 statistically significant compared to cells co-cultured with IL-4 stimulated macrophages.

Figure 4B, indicates apoptotic marker expression in SKOV3 cells following co-culture with untreated and LPS, IL-4 and ME stimulated J774A.1. The SKOV3 cells treated with LPS stimulated macrophages, increased the expression of pro-apoptotic markers (2 to 3 folds) relative to untreated cells and also showed a decrease in anti-apoptotic marker expression. IL-4 stimulated macrophages on the other hand induced a ~ 2-fold increase in the expression of anti-apoptotic markers in SKOV3 cells. With ME stimulation the SKOV3 cells showed an increase in pro-apoptotic and a decrease in anti-apoptotic markers with an expression profile of these markers reflecting the effect observed with LPS stimulated cells (i.e., M1 phenotype).

### Holomonitor analysis of macrophage morphology and motility upon mir-155 treatment and in co-culture with SKOV3 tumor cells

Figure 5 shows the results of the HoloMonitor M4 analysis of some of the co-culture experiments. The holographic images measure the cellular optical thickness, with thicker cells having brighter values in

the gray scales. Two classes of cells have noticeably higher thickness, these being mitotic cells as cells round up, and also macrophages, which have a thicker morphology, regardless of their shape.



**Figure 5:** Holographic imaging and motility patterns of LPS, IL-4 or miR-155 stimulated J774A.1 macrophages co-cultured with SKOV3 ovarian tumor cells.

Panels labeled “A” show macrophage motility plots. Images were segmented using a double Otsu algorithm, which is particularly effective for round cells. Cellular position is recorded for each frame in the analysis. Ten macrophages were selected at the beginning of the analysis, and tracked for the entire 16 hours period. It should be noted that any cell can be selected at any time of the analysis. The cell tracking, color coded for each cell, are shown in a Cartesian Plot, with the starting position in the center, and also overlain on an image of the cells. Panels labeled “B” show a single frame from the analysis, where the morphology of the cells can be seen. Panels labeled “C” show the maximum value Z projection of the 4 dimensional image stack produced in Image J. The brighter objects are the macrophages, and the size of the object is a representation of the area that they covered. Panels labeled “D” are 3 Dimensional representations of the 4 Dimensional plots, also processed in Image J. In practice, these plots are fully rotatable, and allow for close observations of the interactions between the cell types.

In macrophage only cultures stimulated with LPS, the cells often present a bi-lateral symmetry, and a zig-sag shuttling motion, especially early after stimulation. In co culture experiments, we

observed the same shuttling behavior, but it was not as pronounced. In our study SKOV3 ovarian tumor cells were selected as we had observed these to be non-motile except in mitosis (previous unpublished observation on the HoloMonitor). Interestingly we observed motility in our cancer cells, presumably due to interactions with the macrophages.

Macrophages that were only stimulated with IL4 presented radial symmetry, and moved in starburst patterns, often presenting trails that resemble Brussel Sprouts stalks. In the 4-D co-culture plot, the tumor cells trails appeared vertical, with no lateral motion. In the ME treated macrophage culture, many of the cells presented a shuttling behavior similar to that seen in the LPS stimulated cells. There was also a population of cells that presented an angular amoeboid morphology, which is consistent with a transition from radial symmetry to bilateral symmetry. In the 4-dimensional plot, the macrophages presented columns that had an appearance similar to stalagmites as they interacted with the tumor cells. The tumor cells themselves appeared larger, but less dense and became diffuse, possibly as the result of repeated cytolytic hits in the macrophage-induced killing process.

## Discussion

Myeloid derived monocytes and macrophages are highly plastic cells known to express different functional phenotypes in response to microenvironment stimuli. Fully polarized M1 and M2 macrophages are the extremes of these two functional states. LPS stimulated macrophages express the M1 or anti-tumoral/pro-inflammatory phenotype while IL-4 stimulated macrophages express the M2 or pro-tumoral/anti-inflammatory phenotype [20]. The differing polarity of the macrophages leads to altered receptor expression, effector function, cytokine and chemokine production. In tumor tissues, infiltrating macrophages are driven by tumor derived and T-cell derived cytokines to acquire a M2 polarization phenotype [20,21]. MicroRNAs are noncoding RNAs that can regulate the expression of protein-coding genes at post-transcriptional level, which affects cancer and immunity. The levels of several miRs are altered in the two distinct macrophage phenotypes, which exist in the tumor microenvironment [22]. miR-155 is one such microRNAs that is considered to play a critical role in promoting macrophage polarization to the M1 phenotype [22]. Similarly TAM's, which overexpress miR-155, have significantly decreased tumor cell survival, promote tumor cell apoptosis and inhibit tumor cell invasion [23]. Thus miR-based re-polarization of macrophages provides a novel strategy for macrophage mediated cancer therapy. Despite the promise that micro-RNA based therapy holds for the treatment of cancer, several critical hurdles need to be overcome including *in vivo* instability, unsuitable biodistribution, disorder and saturation of RNA machinery and inopportune side effects [16,24]. Several viral vector based systems have been developed to overcome some of the delivery challenges however they have toxicity and immunogenicity-based problems, which limit their clinical usage [16]. Previously we have investigated a safe and effective non-viral vector based solid nanoparticles in emulsion multi-compartmental gene delivery system for successful transfection to macrophages [17].

In this study, we have incorporated miR-155 encoding plasmid DNA in water-in-oil-in-water (WOW) based multiple emulsions systems and assessed the effect on macrophage polarization. We also investigated the effect of these polarized macrophages on SKOV3 human ovarian adenocarcinoma cells both in relation to cellular apoptosis and also in relation to the morphological and motility

differences observed upon co-culture. Firstly we assessed the kinetic effects of LPS and IL-4 stimulation on J774A.1 cells and observed that the M1 and M2 specific markers showed time dependent expression profile post stimulation. Previously, as miR-155 treated bone-derived macrophages had shown modulation of M2 polarized macrophages to the M1 phenotype we investigated the encapsulation and delivery of miR-155 encoding plasmid ME for macrophage re-polarization [22]. The uptake of encapsulated plasmid beginning as early as 1 hour after incubation indicates that the delivery system provides for fast and efficient delivery to the macrophages. Thus this system is an excellent modality for achieving targeted delivery of microRNA and other nucleic acid based therapeutics to macrophages. The expression of miR-155 from the delivered plasmid, induced a phenotype switch in M2 polarized macrophages as evident from M1 and M2 marker expression assessment suggesting that the therapeutic payload is stable and fully functional after formulation.

Investigation into the effect of LPS and IL-4 stimulated macrophages following co-culture with SKOV3 cells, demonstrated M1 phenotypic and M2 phenotypic marker expression respectively, whereas miR-155 treated cells exhibited a phenotype intermediate between the two. Apoptotic marker expression in SKOV3 cells after co-culture with MME stimulated macrophages exhibited induction of apoptosis similar to one seen with LPS (i.e. M1 stimulated macrophages). Thus, the macrophage phenotype switching can be a practical approach to functionally hinder the growth and proliferation of the cancer cells.

Using HoloMonitor based label-free imaging, we have also assessed the morphological and motility behavior of the untreated J774A1 macrophages, and treated with LPS, IL4, Naked Plasmids and ME plasmids, singly and also in co-culture with SKOV3 cancer cells. Morphological changes in macrophages in response to LPS and IL-4 stimulation has been reported in literature, with conflicting results [25,26]. Our results are consistent with those described in reference [26]. However, HoloMonitor based assessment of LPS and IL-4 stimulated cultures suggest links between macrophage morphology and their motility. ME treated macrophages showed motility and morphological profile between the two stimulation states. Thus ME based delivery systems not only provided polarization of macrophages but showed distinct behavior in the presence of the cancer cells. Further studies are warranted to understand the effect of macrophage polarization and presence of cancer cells in real-time to mimic the interaction of these cells as closely as possible to the cells in the tumor microenvironment.

## Conclusions

Tissue-associated macrophages exist in a functional polarization spectrum, with the M2 state being predominately expressed in tumor microenvironment. The M1 phenotype affects decreased tumor cell survival, promote tumor cell apoptosis and inhibit tumor cell invasion. Multiple emulsion mediated miR-155 delivery led to prolonged miR-155 expression and translated to enhanced macrophage polarization. Following co-culture these polarized macrophages enhanced cancer cell apoptosis. Real-time images showed motility and behavior of macrophages between the M2 and M1 stimulated macrophages. Taken together the emulsion based delivery strategy can be a simple solution to ensure delivery to macrophages which in turn serve as Trojan horses capable of homing to TME and demonstrating their anti-tumor effect. This system may be advantageous compared to other non-viral systems that are based on receptor expression or

surface modifications. Non-viral targeted delivery systems based on receptor expression may be compromised if receptor expression is dysregulated in the TME. Additionally, other nanocarrier systems harnessing surface engineering using targeting moieties needs to be extremely specific to prevent uptake by other cell types [27]. In addition to ease of administration and incorporation of multiple payloads, the size dependent targeted delivery to macrophages can be a further advantage over the receptor mediated and surface modification approaches.

Further studies are warranted to test this hypothesis and also to assess cellular interaction rather than only the effect on cytokines and receptor markers to recapitulate the interaction of cells within the complex tumor microenvironment post treatment with microRNA based therapy.

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