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Original Article

Curcumin mediated dendritic cell maturation by modulating cancer associated fibroblasts-derived exosomal miRNA-146a

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ABSTRACT

Background: Though cancer associated fibroblasts (CAFs), being a main component of tumor microenvironment (TME), are known to modulate immune response through secretion of various growth hormones, exosomes carrying miRNAs and cytokines; their effect on dendritic cells (DCs) are yet to be elucidated. Thus, aim of this study was to assess the effect of miRNAs and cytokines released by lung-CAFs and to evaluate immunomodulatory potential of curcumin on DC maturation through modulating their TME.

Material and Methods: To check the effect of CAFs derived exosomes on DC maturation, we cultured imDCs in the presence of CAFs derived conditioned media (CAFs-CM) and characterized by the presence of maturation markers CD80, CD83, CD86 and CTLA4 using qRT-PCR. Additionally, expression of miR-221, miR-222, miR-155, miR-142-3p and miR-146a was assessed to evaluate the role of epigenetic regulators on DC maturation. Likewise, cytokine profiling of CAFs-CM as well as CAFs-CM treated with curcumin was also conducted using ELISA.

Results: Results revealed the generation of regulatory DCs which were characterized by decreased expression of maturation markers in the presence of CAFs-CM. In addition, such DCs showed higher expression of epigenetic regulator miR-146a which was positively correlated with increased expression of anti-inflammatory cytokines like IL-6, IL-10, TGF-β and decreased expression of TNF-α (pro-inflammatory). Moreover, curcumin had the potential to convert regulatory DCs generated by CAFs into mDCs, which were characterized by high expression of co-stimulatory molecules, low expression of CTLA4, lower levels of immune suppressive cytokines production and lower levels of miR-146a.

Conclusion: Collectively, these findings provide insight into understanding the immunomodulatory role of curcumin in targeting CAFs and modulating TME, thus enhancing antitumor immune response in DC based therapy.

KEY WORDS: Cancer-associated fibroblasts, curcumin, dendritic cells, exosomes, immune modulation, tumor microenvironment

INTRODUCTION

Lung cancer is the second most common cancer with high morbidity and mortality in both men and women worldwide. Despite the recent developments in different treatment modalities, rates for the overall survival still remain very low.[1-4] Cancer immunotherapy, the emerging hallmarks of cancer and fourth pillar of current cancer therapy, is emerging as one of the most promising treatment paradigms due to increases in the failure rate of conventional therapeutic regimens such as radio-/chemotherapy and/or surgery.[5,6] Despite promising results in some cancers, immunotherapy has accomplished inadequate success, making it difficult to translate this approach into clinical practice. This could be attributed to several mechanisms which result in immune suppression in cancerous patients.[7] In this regard, a major contributing factor contributing to immune suppression is the influence of the tumor-microenvironment (TME). Of all the different cell types, cancer-associated fibroblasts (CAFs) are the most abundant stromal cells in the TME, with numerous reports implicating their important role in regulating almost all of the hallmarks of

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cancer.[8-12] Moreover recent studies report on their potential to suppress patients' immune system by various mechanisms, including for example, downregulation of antigen presentation, elevated expression of surface immune-inhibitory molecules, and secretion of soluble immunosuppressive factors (i.e., cytokines, chemokines, growth factors and very recently reported exosomes, etc.).[13-19] In considering such roles, CAFs have received intensive interest as a target and tool in various cancers as well as in diagnosis. However, presently little is known as to how CAFs regulate anti-tumor immune responses in various solid malignancies. In this regard, our focus here is on exosomes that govern the paracrine interaction within TME to provide new insights into their immune evasion mechanisms.

Exosomes are small vesicles (30-100 nm in diameter) with several unique properties. They carry enormous information in the form of bioactive molecules, such as mRNAs, microRNAs (miRNAs), and long non-coding RNAs (lncRNAs); genomic DNA, cDNA, and mitochondrial DNA (mtDNA); and proteins and lipids.[20-22] Most of the current studies are focusing on tumor cell-derived exosome; however, little is known about CAFs-derived exosomes (CDEs) and their influence on immune suppression in dendritic cell-based immunotherapy. Although it has been shown that CAFs can induce immunosuppressive TME, the contribution of CDEs in this phenomenon, if any, is yet to be elucidated. Besides, exosomal miRNAs are known to induce post-transcriptional regulatory mechanisms resulting in epigenetic changes in the recipient cells.[23,24] Immunotherapy, despite being promising treatment approach, has till now failed to eliminate cancer cells from the patient's body, this relating to the potential of CAFs-derived exosomes' immunosuppressive tumor-promoting microenvironment. Moreover, as the present treatment approach focuses only on targeting cancerous cells is insufficient to combat aggressive cancer; a synergistic treatment strategy is required to target TME in order to develop more potent DC-based immunotherapy.

Curcumin, traditionally known for its anti-inflammatory effects, has been shown to be a potent immunomodulatory agent that can modulate the activation of various immune cells by downregulating pro-inflammatory cytokines, including IL-1, IL-2, IL-6, IL-8, IL-12.[25-28] In our previous studies, we have reported on its different potential to target cancerous cells in Acute myeloid leukemic (AML) patients[29] as well as lung cancer patients.[30] Thus, the aim of this study was to investigate the role of CDEs on the maturation of DCs by evaluating the expression of various miRNAs involved in it, as well as concentration of various pro-tumorigenic and anti-tumorigenic cytokines in CAFs-derived conditioned media [Figure 1]. Also evaluated here, was the immunomodulatory potential of curcumin on DC maturation by targeting lung cancer patients'-derived CAFs (Lung-CAFs) through modulating their TME.

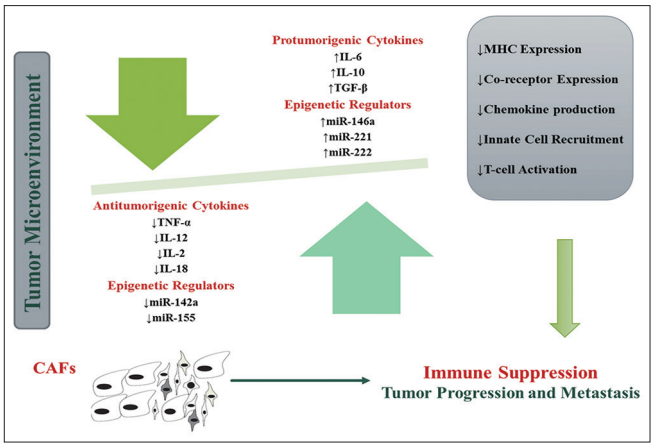


Figure 1: Graphical representation of the effect of tumor-microenvironment on immune suppression. CAFs present in TME secrete various cytokines and exosomes bound miRNAs, eventually resulting into immune suppression via decreased MHC expression, pro-tumorigenic chemokine production, T-cell activation, etc

MATERIALS AND METHODS

CAFs isolation and cell culture

Tumor samples (biopsy) from lung adenocarcinoma patients were obtained from our institute. HIV and HbsAg positive patients were excluded from the study. Experimental subjects were randomized as basic criterion, that is, demographics like age, sex (Male/Female), size, weight, etc., were not assessed. Subject blinding and power analysis were not necessary in this study. Written informed consents were obtained from the patients as well as healthy individuals. This study was approved by the Institutional Review Committee of Institute. CAFs from lung adenocarcinomas and Normal Fibroblast (NF) were isolated and cultured in DMEM media, supplemented with 10% FBS at 37°C in a humidified incubator in 5% CO_2.

Characterization of CAFs

Western blot analysis was done to characterize the isolated CAFs. Cells were collected in RIPA lysis buffer (150 mM NaCl, 0.5% Nonidet P-40, 1% Triton X-100, 0.5 mM EDTA, 0.1% SDS, 50 mM Tris-HCl, pH 7.6), containing protease inhibitor cocktail (Sigma-Aldrich), after washing with ice cold PBS. Protein extracts were quantified by the Bradford Protein Assay. For Western-blot analysis, 50 mg of total proteins were resolved on 12% BIS-TRIS gels (SDS-PAGE) and transferred to nitrocellulose membrane (Biorad) in Bicine transfer buffer by electroblotting at 50 V for 2 hours. After electrophoresis, the membrane was stained with cypro ruby (Sigma-Aldrich) and blocked with TBST (TBS buffer saline with 0.1% Tween-20) containing 1% BSA for 15 min and then incubated overnight with primary antibodies (vimentin, α-SMA & FAP; Abcam). After washing with TBST, membranes were probed for 1 h at room temperature with secondary antibody. Finally, chemiluminescence was used to detect the labeled proteins and the signals were quantified using a Gel-Dock β-actin was used as an internal control.

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Collection of conditioned media

CAFs and NFs were allowed to grow till 90-95% confluency and then washed in PBS to remove FBS. Cells were then grown in serum free medium for 48 hrs to condition the medium; and supernatants were collected after this. For curcumin treated CAFs' (CurCAFs)-derived conditioned media, Lung-CAFs were allowed to grow in the presence of 10 μM curcumin for 48 hrs and supernatants were collected and filtered through 0.22 μm filters to remove debris and further used as conditioned media.

Quantification and sizing of conditioned media-derived extracellular vesicles (EVs)

Extracellular vesicle (EV) enumeration and size distribution was carried out using the NS300 (NanoSight Ltd., Amesbury, UK) following manufacturer's instructions. The instrument uses a laser light (405 nm) source to illuminate nanoscale particles which are seen as individual point-scatters moving under Brownian motion. The path of the point scatters, or particles are calculated over a 60 sec time frame to determine their velocity and size distribution using image analysis NTA software (version 2.3; Nano-Sight Ltd.).

Human CD14+ monocyte differentiation

Peripheral blood Mono Nuclear Cells (MNCs) were isolated from patients by density gradient centrifugation using Histopaque-1077 (Sigma) and CD14+ cells were isolated using the Human CD14 positive selection kit (StemCell Technologies). Cells were cultured in RPMI-1640 media supplemented with 10% FBS. rIL-4 (50 ng/mL, StemCell Technologies) and rGM-CSF (50 ng/mL, StemCell Technologies) were added to culture media every alternate day for differentiation of monocytes to immature DCs. At day 6, immature DCs were harvested and cultured in 2 ml of CAFs, NFs and CurCAFs-derived conditioned media. Furthermore, cells were also stimulated with cell lysates prepared from isolated CSCs to prepare mDC, CAF-mDCs, NF-mDCs, and CurCAF-mDCs for 48 hrs.

RNA/Exosomal miRNA isolation and real-time PCR

Total endogenous mRNA from different sets of mDCs were isolated using a NucleoSpin RNA kit (MACHEREY-NAGEL) according to the manufacturer's instructions to detect the expression of maturation-associated markers, such as CD80, CD83, CD86, and CTLA4. A total of 1 μg of mRNA was used to generate cDNA and further, this cDNA was used as a template for PCR as described previously.[31] Exosomal miRNA was isolated from 2 ml of conditioned media as described by Glynn *et al.*[32] and miRNA specific cDNA was produced using first strand miRNA specific cDNA kit (Agilent technology) following the manufacturer's protocol. A PCR reaction mixture was prepared as mentioned above.

Cytokine assay

Conditioned media was harvested for IL-6, IL-10, TGF-β, and TNF-α concentration measurements in different subsets of DCs, using the Bio-Plex Suspension Array System (Bio-Rad).

Statistical analysis

Results were expressed as mean ± SEM (Standard error of the mean) and differences between experimental and control groups were assessed by Student's *t* test. Statistical analysis was performed using online free platform. *P* values <0.05 were considered as statistically significant.

RESULTS

Identification and characterization of CAFs isolated from lung adenocarcinomas

Lung-CAFs were characterized using their specific markers α-SMA, FAP, and vimentin by western blot analysis. Results showed high expression levels of these markers in Lung-CAFs, as compared to NFs [Figure 2]. α-SMA+ expression was considered characteristic of Lung-CAFs, while α-SMA- expression was considered representative of NFs.

Characterization of the exosomes derived from conditioned media

To elucidate the role of exosomes in generating regulatory DCs during their maturation process *in-vitro*, exosomes were isolated from the conditioned media; and their presence was confirmed by nanoparticle tracking analysis using Nanosight. This revealed that isolated exosomes were approximately ~43 nm in size, confirming their presence in the conditioned media derived from Lung-CAFs [Figure 3].

Lung-CAFs inhibit phenotypic maturation of imDCs

Furthermore, we determined any DC phenotypic changes in imDCs, mDCs, NF-DCs, and CAF-DCs by real-time PCR, using CD80, CD83, CD86, and CTLA-4 markers associated with DC maturation status. As shown in [Figure 4], CAF-DCs articulated lower expression of functional markers, such as

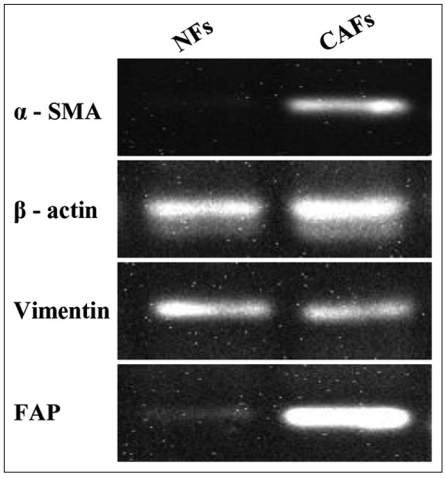


Figure 2: Western blot analysis of CAFs marker. Results showed differences in expression of α-SMA and FAP between NFs and CAFs; while there was no significant difference was observed in Vimentin expression. Vimentin (anti-rabit, 1:500 dilution), α-SMA (anti-rabit, 1:500 dilution), FAP (anti-rabit, 1:1000 dilution), secondary antibody (1:10,000 dilution)

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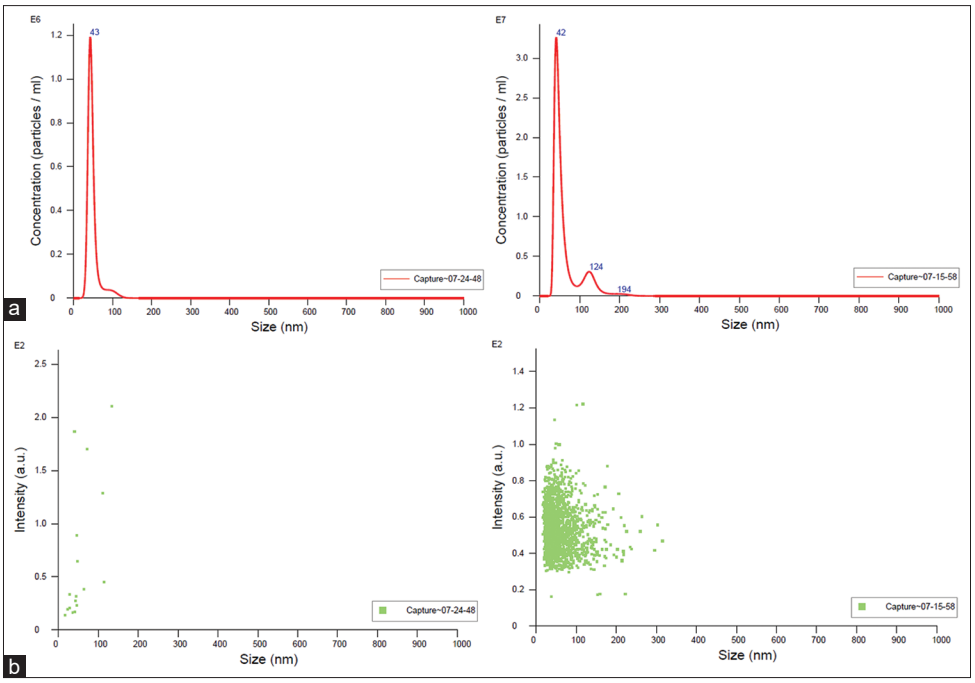


Figure 3: Characterization of conditioned media-derived extracellular vesicles. Nanosight results confirmed the presence of exosome as their sizes are in the range of 30-100 nm. (a) Graphical representation of average concentration (particles/ml)/size. (b) Graphical representation of particles intensity/size

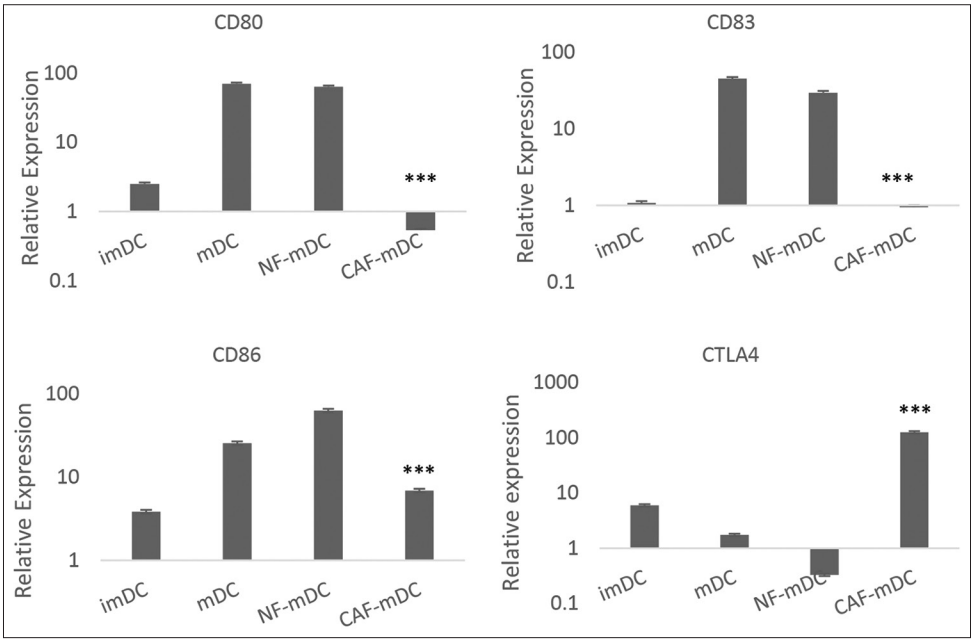


Figure 4: CAFs educated DCs to acquire tolerogenic properties. Maturation markers CD80, CD83, CD86, and CTLA4 were evaluated by real time PCR. Data indicate the mean ± SEM of different experiments. Data was normalized against β-actin and compared to CD14+ cells. ****P* value <0.0001

mature molecule CD83 and the co-stimulatory molecules CD80 and CD86, as compared to mDCs that were cultured alone, as well as NF-DCs; while expression of CTLA4 the immune checkpoint marker, was shown to be increased in CAF-DCs when cultured in the presence of CAF-CM, suggestive of the fact that they might be associated with their regulatory functions. Moreover, there were no significant differences in the expression of these molecules associated with DC maturation between mDCs and NF-DCs; while there was a significant difference in their expression between CAF-DCs, as compared to NF-DCs and mDCs. These results demonstrated the immunomodulatory potential of Lung-CAFs in recruiting the normal DCs to regulatory/tolerogenic DCs.

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CAF-mDCs revealed tolerogenic characteristics

Considering that CAF-mDCs downregulated the expression of markers associated with functionality of DC maturation process, it was hypothesized that they might possess immunosuppressive potential. To further define their tolerogenic characteristics, their cytokine profile of conditioned media was determined by enzyme-linked immunosorbent assay (ELISA). Results demonstrated that CAF-mDCs were inclined to express more immunosuppressive cytokines, such as IL-6, IL-10, TGF-β, and less TNF-α [Figure 5]. Unlike CAF-mDCs, NF-mDCs did not exhibit the same immunosuppressive characteristics, suggesting that those effects only occurred in the presence of CAF-derived conditioned media, while generating mDCs. This confirmed here that CAF-mDCs have a potent ability to convert imDCs to regulatory DCs having immunosuppressive ability in that TME during immune reactions in lung cancer.

CAFs-CM enhances the expression of epigenetic regulator miRNA-146a

Initially, we evaluated the expressions of various miRNAs (epigenetic regulators), for example- miR-34, miR-221, miR-222, miR-142, and mir-146a which are associated with the DC maturation process. The expression level of miR-146a was dramatically increased as compared to other miRNAs, and thus, we considered miRNA-146a for further evaluation. To study the changes in expression levels of miRNA-146a in conditioned media derived from CAFs generated DCs *in-vitro*, miRNA enriched total RNA was isolated and performed qRT-PCR for miRNA expression analysis. The fold change in miRNA expression higher than two-folds were considered statistically significant. As shown in Figure 6, the expression levels of miRNA-146a were drastically upregulated in CAF-mDC (mDCs cultured in the presence of CAFs-CM) as compared to mDC as well as NF-mDC, suggesting its ability to suppress function and maturation of DCs.

Modulatory effect of curcumin on maturation markers of DCs

After evaluating various anti-cancer activities of curcumin in previous studies,[29,30] we evaluated curcumin's potential to modulate the markers (CD80, CD83, CD86, and CTLA-4) involved in the DC maturation process. Our findings disclosed a significantly enhanced the expression of CD80, CD83, and CD86 markers ($p < 0.0001$), with a significantly decreased the expression of CTLA4 ($p < 0.0001$) when imDCs were cultured with curcumin treated CAFs-derived conditioned media (CurCAF-mDC) [Figure 7a].

Curcumin-induced modulation of tumor microenvironment

Considering that curcumin upregulated the expression of maturation and functional markers of DCs, we evaluated the effect of curcumin on the pro-inflammatory cytokines (IL-6, IL-10, and TGF-β) and the anti-inflammatory cytokine (TNF-α), released by Lung-CAFs. Here, in the presence of curcumin, the concentration of IL-6, IL-10, and TGF-β was significantly ($p < 0.0001$) decreased, while no significant difference was observed in TNF-α levels [Figure 7b]. These findings indicate that curcumin mediates a modulatory effect on CAFs in their TME.

Effect of curcumin on epigenetic regulator (miRNA-146a) of DC maturation

Furthermore, we investigate the inhibitory potential of curcumin on miRNA-146a expression, which is bound in the form of exosomes in conditioned media derived from CAFs. miRNA-146a

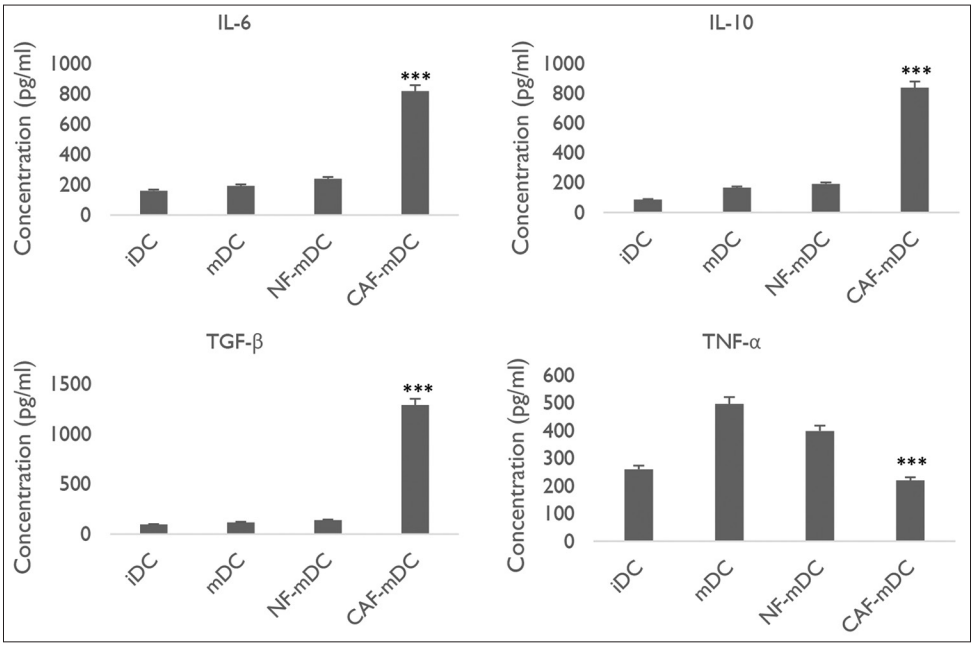


Figure 5: CAF-mDCs exhibit tolerogenic characteristics. Cells supernatants were collected for IL-6, IL-10, TGF-β, and TNF-α concentration measurements. The data indicate mean ± SEM of five different experiments

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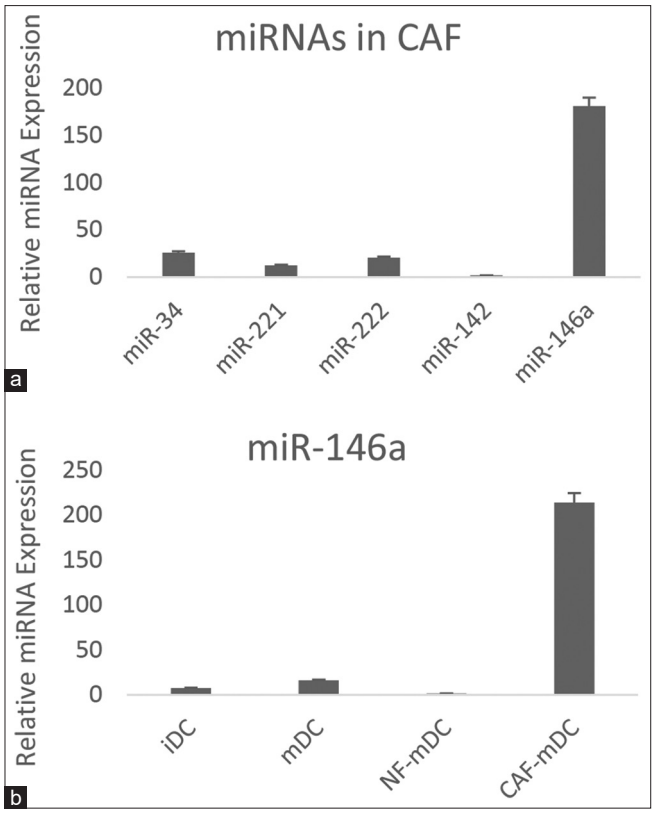


Figure 6: Effects of CAFs-CM on the expression of miRNA-146a in CD14 + monocytes derived DCs. (a) miR-34, miR-221, miR-222, miR-142, and miR-146a expression in CAFs-CM. (b) The expression of miRNA-146a was determined by qRT-PCR and results were analyzed using 2^{-ddCt} method. The data indicate mean ± SEM of five different experiments (***$p < 0.0001$)

expression significantly decreased ($p < 0.0001$) when mDCs were generated from imDCs in the presence of curCAFs-derived conditioned media [Figure 7c]. This implies that curcumin may potentially regulate the epigenetic mechanisms controlling the various functions of DCs, resulting in the enhanced efficacy of DC-based immunotherapy.

DISCUSSION

Although it is known that CAFs possess the capacity to induce immunomodulation, how they influence the DC maturation and its function still remains undetermined. In the present study, we observed that CAFs derived from lung adenocarcinomas induce the acquisition of a tolerogenic phenotype by DCs, through the secretion of various pro-tumorigenic cytokines, such as IL-6, IL-10, and TGF-β; and miRNA-146a released through exosomes.

Firstly, we have shown that exosomes released in conditioned media derived from lung-CAFs promote the differentiation of imDCs to regulatory DCs leading toward immunosuppression. Results demonstrated that there was a decrease in the expression levels of markers associated with maturation of DCs, including CD80, CD83, and CD86 in CAF-mDC, as compared to mDC and NF-mDC, suggesting the inhibitory effect of CDEs in their TME (i.e., conditioned media) on the DC differentiation and maturation process. On the other hand, the expression of the Treg-associated marker CTLA-4 was observed to be increased, which notably is involved in the production of regulatory DCs via suppression of T-cell response, through IL-10 and IDO production.[33]

Though, there are several reports revealing the importance of exosomes in cell-to-cell communication and resulting into the metastasis by inducing drug resistance[21]; their role in modulating cellular immunity still requires elucidation. In a review article, Sinha *et al.*[34] discussed various aspects of exosomes, including their biogenesis, epigenetic regulation, TME modulation, immune evasion, drug resistance; all of which eventually lead to tumor progression and metastasis. Several studies have reported that exosomes can mediate cancer progression by releasing miRNAs. miR-10b modulated tumor promoting microenvironment in breast cancer[35] and release of exosomal miR-1245 propagated tumor progression by modulating macrophages.[36] Similarly, it has also been reported that exosomal miR-105, miR-939, and miR-181c induced metastasis in breast and brain cancer.[37] Thus, in this study, we focused on the effect of miRNAs released in the form of exosomes by CAFs, on the maturation process of DCs. In addition, previous studies have shown that mirRNA-146a regulates gene expression in different cell types of the immune system and is an important factor in the regulation of the innate immune response and inflammatory disease, which is induced in response to cytokines.[38,39] In hepatocellular carcinoma (HCC), exosomal miR-146a-5p promoted M2 polarization.[40] In this study, we found that expression of miRNA-146a was markedly increased in the presence of CAFs-CM treated mDCs, as compared to NFs-CM. These findings clearly demonstrated the role of epigenetic regulator miRNA-146a in inhibiting CAFs-CM treated mDC differentiation and maturation; and which is similar to several other studies. Du *et al.*[41] suggested that aberrant miRNA-146a expression was one of the key factors responsible for the inhibition of DC maturation and antigen presentation function, and that this inhibitory effect on DCs might be due to the repression of Smad4 mediated signaling pathway by BxPC-3 conditioned media. Apart from this, numerous studies have reported on the importance of exosomes in treating cancers. Hosseini *et al.*[42] provided an insight into tumor-derived exosomes-induced subversion of DCs differentiation, maturation, and function. Whiteside *et al.*[43] discussed how they can be used as biomarker as a means of liquid biopsy to monitor disease progression and/or response to various treatments. Xu *et al.*[44] described several ongoing exosome-based immunotherapy clinical trials and their implications.

Furthermore, here we discovered the cytokine profile of conditioned media derived from different sets of DCs, wherein there was an elevated expression of pro-tumorigenic cytokines like IL-6, IL-10, and TGF-β and a decreased expression

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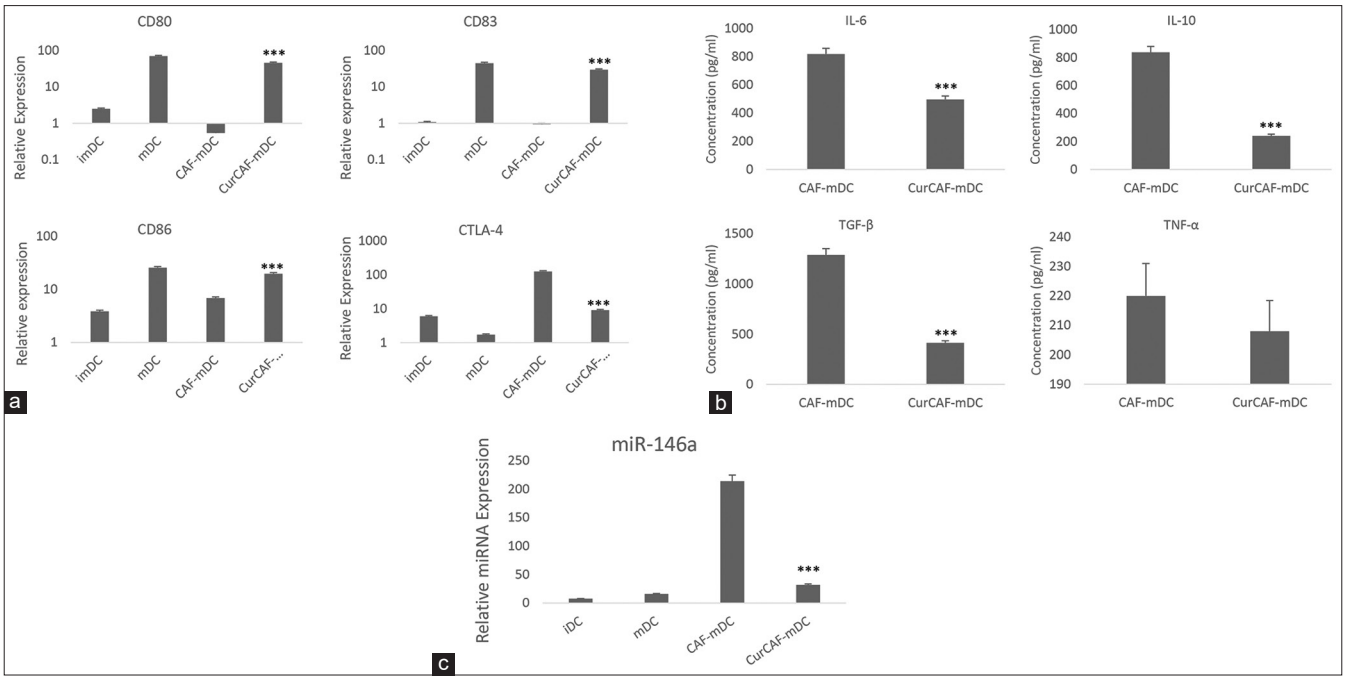


Figure 7: (a) Effect of curcumin on maturation markers of DC. CD80, CD83, and CD86 markers were significantly upregulated as compared to CAF-mDC when cultured in the presence of curcumin, while CTLA4 was significantly downregulated in the presence of curcumin. Data indicate the mean ± SEM of different experiments. ****P* value <0.0001. (b) Effect of curcumin on cytokines in conditioned media of CAFs. Curcumin was shown to decrease the concentration of anti-inflammatory cytokines like IL-6, IL-10, and TGF-β as compared to untreated CAFs-derived conditioned media. Data indicate the mean ± SEM of different experiments. ****P* value <0.0001. (c) Effect of curcumin on miR-146a. Results demonstrated that expression of miR-146a was significantly downregulated in the presence of curcumin treated CAF-derived conditioned media as compared to CAF-mDC. Data indicate the mean ± SEM of different experiments. ****P* value <0.0001

of TNF-α, an anti-tumorigenic cytokine; which reflects the results of Cheng *et al.*[45] who suggested that hepatic carcinoma-associated fibroblasts (hCAFs) have the potential to induce IDO-producing regulatory dendritic cells through IL-6-mediated STAT3 activation. Further, there are reports suggesting the involvement of these cytokines in the inhibition of immune response. In this regard, IL-10 has been described as the main immunomodulatory cytokine by various groups. Arce-Sillas *et al.*[46] showed that IL-10 favors a tolerogenic phenotype which promotes the production of further IL-10 in DCs. TGF-β is a multifunctional cytokine having different effects on cells. It has potential to regulate several types of immune cells by various mechanisms, including inhibition of DCs functional activities.[47] It also promotes the differentiation of naïve T cells into Treg cells by inducing FOXP3 expression, or into Th17 cells in the presence of IL-6.[48] Additionally in this study, we also have reported on the elevated levels of these cytokines, this being suggestive of their immunosuppressive effects on the DC maturation process.

As we have discussed the importance of various nutraceuticals as a treatment approach to modulate immune responses in patients by regulating their epigenetic mechanisms,[49] we focused on curcumin to ascertain its immune-modulatory potential. In this study, curCAF-mDC showed significantly increased expression of maturation markers (CD80, CD83, and CD86) and decreased expression of CTLA4 marker, as compared to CAF-mDC after curcumin treatment, suggesting its potential to educate regulatory DCs toward the mDCs pathway. Importantly, this would eventually result in an increased immunity against cancer antigens. Moreover, several reports have suggested that curcumin-treated DCs produce significantly lower levels of IL-6, IL-12, IL-10, and TNFα, thus creating a Th2 permissive environment.[50] In addition, there are several other reports confirmed that curcumin inhibited IL-1b, IL-6 through modulation of NF-kB and MAPK pathways,[51] IL-2,[52] IL-5,[53] IL-8,[54,55] IL-12 induced STAT4 phosphorylation,[56] and IL-18[57] expression. Moreover, curcumin-inhibited IL-6-induced STAT3 phosphorylation and nuclear translocation in multiple myeloma cells.[58] Recently, Paul S and Sa G revealed that curcumin acting as an adjuvant in immunotherapies may present with novel approaches for researchers and clinicians to obtain improved treatment outcomes.[59]

Collectively, our results concur with previous findings reported by other researchers, signifying curcumin's potential to decrease the secretion of IL-6, IL-10, and TGF-β in conditioned media, this being mediated via curcumin's efficacy to instruct CAFs to secrete exosomes resulting in the creation of an immunomodulating TME from an immunosuppressive TME environment. As we discussed above, the main hurdle in failure of DC based immunotherapy is involvement of TME; it is important to target that immune-suppressive TME along with DC based immunotherapy to increase its efficacy. Thus,

this study may provide basis to revise fundamental treatment rationales and formulate synergistic therapeutic approaches by using curcumin along with DC-based immunotherapies to overcome cellular resistance in cancer treatment but it still requires more in-depth research to have an in-sight into mechanism, which might open up a newer avenues for curcumin's use as an appropriate immunotherapeutic modulator.

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Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form, the patient(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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Conflicts of interest

There are no conflicts of interest.

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