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TUMOR MICROENVIRONMENT-INDUCED ALTERATION IN MORPHOLOGICAL PARAMETERS, ADHERENCE AND MULTINUCLEATED GIANT CELL FORMATION IN MURINE PERITONEAL MACROPHAGES

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Running Head: DL-induced morphological alteration in murine macrophages.

Abstract

Macrophages are mononuclear phagocytes, have an important role in innate and adaptive immunity against microbial invasion and neoplastic growth. Microbial components as well as tumor antigens can activate macrophages to release various effector molecules such as RNI, ROI, cytokines, chemokines, and growth factors aimed at destruction and/or killing transformed cells or cells carrying infectious agents. Compelling evidence suggests that tumor cells release several immunosuppressive factors that lead to infiltration of various immune cell populations including at the tumor microenvironment. The exposure of immunosuppressive microenvironment leads to M2 macrophage polarization in the host which are known to be a poor antigen presenting cells, secrete immunoregulatory cytokines, and impair cell-mediated immune response leading to uninterrupted tumor growth and progression.Though, much works have been done to show the functional changes in macrophages in tumor microenvironment, but in vitro study comprising role of tumor/tumor microenvironment on morphological alterations has not been done. Therefore, in the present study, we investigated the effect of Dalton's lymphoma cells and/or DL conditioned medium on the morphological alterations of peritoneal macrophages, their adherence to the glass slides, and multinucleated giant cell formation.

Key words : Dalton's lymphoma •Macrophages •Morphology •Adherence • MNGC

Abbreviations: DL, Dalton's lymphoma; DLCM, DL cell conditioned medium; MNGC, Multinucleated giant cells; PMA, Phorbol-12-myristate-13-acetate;

IL, Interleukin; MAF, Macrophage activating factor; GM-CSF, Granulocyte monocyte-colony stimulating factor; TAMs, Tumor-associated macrophages.

1. Introduction

Macrophages are differentiated tissue phase of a mononuclear phagocyte system which plays an essential role in immunity against pathogen invasion and neoplastic growth occurring in tissue [1]. They are heterogeneous population of cells and show distinct character and functions specific to their location [2]. Their tissue specific distinctive morphological differences within and among macrophage population has also been attributed to heterogeneity of macrophages [3]. They are distributed in all organs and tissues after birth. The macrophages can be readily differentiated from other immune cell types on the basis of morphologic features, metabolic pathways, physical characteristics, and phagocytic properties. These characteristics can be further implied to differentiate the macrophages from different anatomic locations as well as normal resident macrophages with that of activated macrophages [4, 5]. On the other hand functional differences can be readily distinguished by studying adherence to glass, and phagocytosis of yeast cells and immune complexes and other standard method [6]. Furthermore, normal resident and activated macrophages from the same anatomic location have been shown to exhibit distinct characteristics that can be readily identified [7, 8].

Morphological study of macrophages on Wright-stained films indicates that most macrophages are oval and 15 to 25 μm in diameter with an eccentrically placed remiform or fusiform nucleus containing one or two distinct nuclei and finely dispersed nuclear chromatin. Electron microscopic study suggests the presence of prominent membrane ruffles or microvilli which undoubtedly increases surface area and phagocytic/pinocytic credential of macrophages. However, it has been shown that these morphological features are not constant; these distinct morphological features are quite dependent on the microenvironment in which they resideand/or dependent to its functional states.Macrophage activation promotes increase in mean cell size, cytoplasmic granularity and volume-to-surface ratio [9, 10]. Further, activated macrophages or macrophage activated with pathogens show enhanced membrane ruffles, enhanced cytoplasmic extensions, and increased adherence to the glass surface or other substratum [11, 12] that reflects its functional requirements at the site aimed at removing the pathogens.

In a recent study, it has been shown that cadmium chloride can cause significant morphological alterations in the testicular macrophages characterized by reduction adhesive property of the macrophages [13]. Phorbol-12-myristate-13-acetate (PMA) has also been known to modulate the morphology of macrophages that are associated with intracellular calcium metabolism [14]. In addition, many agents have been reported to induce significant morphological changes in the macrophages that includeproteolytic enzymes, acidic conditions, glassware covered with antigen-antibody complexes [15], ATP [16], and macrophage activating factors. (MAFs) [17].

Proteases of the alternative pathway of complement activation have been demonstrated to induce spreading of human monocytes on glass slides. In addition, tissue culture conditions such as temperature, osmolarity, cations, or serum present in the medium affect the spreading of macrophages [18, 19].

Macrophages from cancer patients have been reported to show less elaboration of surface features and reduced nuclear, nucleolar and mitochondrial and grew less compared to macrophages from normal subjects [20] which suggests that tumor microenvironment is well involvement in determining the morphological features of macrophages. Tumor microenvironment contains various anti-inflammatory cytokines and immunosuppressive factors secreted by tumor cell that might be responsible for tumor-mediated morphological alterations in the macrophages [21]. IL-4 and GM-CSF have been demonstrated to induce macrophage-macrophage homotypic fusion and therefore multinucleated giant cell formation in the macrophages [22]. Interferon gamma (IFN-γ) has been shown to enhance macrophage spreading on glass surface and increase N:C ration and roundness of the macrophages [19, 23]. However, detail study regarding the effect of tumor growth and progression on the morphological alterations in various other tumor models is not available.

Therefore, in this chapter, we sought to show the effect of DL cell conditioned medium (DLCM) on the morphological alteration, adhesion and multinucleate giant cell formation in the macrophages. It was, for the first time, observed that the DL cell conditioned medium results in the significant changes in external morphology of macrophages characterized by loss of membrane ruffles and cytoplasmic extensions, suppressed adhesion to substratum and enhanced MNGC formations.

2. Materials and Methods

2.1. Mice and tumor system

Inbred populations of BALB/c (H^{2d}) strain of mice of either sex were used at 8-12 weeks of age. All animals were kept in conventional cages (6 animals in each cage) and received unsterilized food and water *ad libitum*. Experimental animals were inspected daily for survival. All animals were kept and maintained in utmost care under the guidelines of Animal Ethical Committee, Banaras Hindu University.

For tumor system, healthy mice of either sex at 8 to 12 weeks of age were injected intraperitoneally (*i.p.*) with 1.0×10^6 DL cells in 0.5ml sterile PBS. The DL cells for transplantation were obtained from ascitic fluid of DL-bearing mice, where the yield of the cells is higher and maintained in ascitic form *in vivo* by serial transplantation.

2.2. Reagents

Tissue culture mediumRPMI1640 from Hi Media (India) and foetal calf serum (FCS) was purchased from Invitrogen, Grand Island, NY, USA. Anti-mice antibody for IL-4 and IFN-γ were obtained from R&D Systems, Abingdon, United Kingdom.

Phorbol 12-Myristate 13-Acetate **(**PMA) was procured from Sigma chemicals, Bangalore, India.Na_2HPO_4, KH_2PO_4, formaldehyde and trypsin were purchased from Qualigens, Mumbai, India. Acetone was obtained from Rankem Ltd., Mumbai, India. Gluteraldehydewas purchased from Serva Electrophoresis, Heidelberg, Germany. All other chemicals otherwise stated were obtained from Qualigens, Mumbai, India.

2.3. Preparation DL cell-conditioned medium

DL cells were isolated from full grown DL-bearing mice. The peritoneal cavity of mice was injected with 5ml phosphate buffer saline solution (PBS) with the help of 5ml syringe with 24 gauge needle. After that, peritoneal exudate cells were aspirated. Cells were washed three times with chilled phosphate buffer saline and centrifuged at 1000 rpm for 5 min. Cell pellet was collected by discarding the supernatant and cultured in complete RPMI1640 media in CO_2 incubator at 37°C and 5% CO_2 concentration to have healthy numbers of DL cells. After regular interval, small amount of complete media was added to the DL suspension. Cells were centrifuged at 3000 rpm for 10 min, supernatant were collected and filtered by 0.2µm membrane and elute were stored at 4°C in aseptic condition.

2.4. Isolation and activation of peritoneal macrophages

Macrophages from healthy mice of 8-12 weeks of age were prepared by a standard method. Peritoneal exudate cells (PEC) were harvested by peritoneal lavage using chilled serum-free culture medium RPMI1640. The PECs were then transferred into a vented plastic tissue culture flask (Tarson, Kolkata, India) for culture at 37°C in CO_2 incubator (Sheldon, Cornelius, OR, USA). The non-adherent cells were discarded by washing three times with lukewarm serum-free culture medium with gentle flushing. More than 95% of the adherent cell population was that of macrophages, as determined by morphology. The macrophages were then collected from the tissue culture flask using a cell-scraper (Corning Inc., Corning, NY, USA), resuspended in serum-free culture medium. Cells were counted and seeded in culture plates (Tarson, Kolkata, India) at a cell density of 1×10^6 in conditioned medium for 24 hrs in CO_2 incubator at 37°C at 5% CO_2 and PMA at the concentration of 10µg/ml.

2.5. Isolation and culture of DL cells

DL cells were isolated from full grown DL-bearing mice. The peritoneal cavity of mice was injected with 2-3ml phosphate buffer saline solution (PBS) with the help of 5ml syringe with 24 gauge needle. After that, peritoneal exudate cells were pulled out without any peritoneal lavaging, and washed three times with chilled phosphate buffer saline and centrifuged the cell suspension at 1000 rpm for 5 min. Cell pellet was collected by discarding the supernatant and cultured in complete RPMI1640 media in CO_2 incubator at 37°C and 5% CO_2 concentration to have healthy numbers of DL cells.

2.6. Double staining of macrophages

Double staining of normal peritoneal macrophages and tumor-associated macrophages were performed by a standard method. Briefly, isolated and purified peritoneal macrophage were adhered to the glass slide for 2 hr and then fixed in 100% methanol for 1 min. Fixed macrophage were placed in hematoxylin (BD Chemicals, Greenwood, CO, USA) for 10 min then dehydrated into graded ethanol up to 70% ethanol. Slides were then counterstained with freshly prepared alcoholic eosin solution (BD Chemicals, Greenwood, CO, USA) for 5 min thereafter mounted in DPX and examined under light microscope.

2.7. Scanning electron microscopy

Samples for scanning electron microscopy (SEM) were prepared according to the method of Tameike et al., 1996 [24]. Normal peritoneal resident macrophages (NMO) and tumor-associated macrophages (TAMs) were fixed in 2.5%glutaraldehyde prepared in 0.1M sterile PBS (137mM NaCl, 8.1mM $Na_2HPO_4.12H_2O$, 2.68mM KCl, 1.47mM KH_2PO_4) for 30 min and post fixed with 1% OsO_4 overnight. After fixation, they were dehydrated using ethanol series. Samples were coated by gold-palladium (Quantum technology-SC7620), and observed using a Scanning electron microscope (Zieas-EVO LS-10) at 25 kV at LV mode from central facility of Department of Zoology, BHU, Varanasi, India.

2.8. Adhesion assay

Four wells cell culture plates were coated by incubating them with murine albumin at 10mg/ml diluted in RPMI1640 or DL cell conditioned medium for 30 min to 60 min at 37°C in an atmosphere of 5% CO_2 and 95% air. The coating suspension was removed, the wells gently rinsed with phosphate buffered saline (PBS)-ethylenediamine tetra-acetic acid (EDTA) and airdried. Wells incubated with RPMI1640onlywere used as controls while other wells were incubated in RPMI1640 and stimulated with PMA or incubated with DL cell conditioned medium only. The coated wells were then incubated with 500µL (0.5×10^6 cells) of the original cell suspension under culture conditions for 5, 15 and 60 min. The non-adherent cells were counted and calculated to get percent adhesion as follows:

$$\textbf{Percent adhesion} = \left(\frac{\textbf{TotalNo. of cells} - \textbf{nonadherentcells}}{\textbf{Totalno. of cells}}\right) \times \mathbf{100}$$

2.9. Macrophage giant cell formation and determination of the fusion Index

Macrophage cell suspensions (1×10^6 cells/ml) were dispended as a 50µl droplet in the centre of a well in 96 well flat-bottomed plates to produce a dense monolayer of NMO and TAMs separately and incubated for 6, 12 and 24 hrs at 37°C under 5% CO_2 in CO_2 incubator. Cells were analyzed for clustering and fusion after different time interval using inverted microscope (Olympus CKX-41; Olympus Imaging, River Valley Road, Singapore), after that culture medium was removed from

the well, cell were fixed and stained with hematoxylin and eosin and observed under microscope.

The fusion index (FI) of macrophages was determined by counting the number of stained nuclei in MGC and the total number of nuclei within a given field under a microscope at 1000 × magnification as earlier reported by McInnes and Rennick, 1988 [25] by following formula:

$$\boldsymbol{FI}(\%) = \frac{\boldsymbol{No.ofnucleiwithinMGC}}{\boldsymbol{Totalno.ofnucleicounted}} \times \mathbf{100}$$

2.10. Statistical Analysis

All the experiments were performed at least three times and data were taken as significant at $p<0.05$. The statistical analysis was performed by using unpaired Student's t-test. All statistical analysis was performed on statistical software package Sigma Plot version 11.0 (Systat Software Inc., CA, USA).

3. Results

3.1. Morphological characterization of normal peritoneal macrophages and tumor-associated macrophages

Macrophage harvested from normal healthy and DL-bearing mice were incubated for 1 hr under culture condition (at 37°C in an atmosphere of 5% CO_2 and 95% air) and the external morphology was assessed by Light Microscopy and Scanning Electron Microscopy. The macrophages harvested from normal healthy mice appeared to be very heterogeneous and varied in shape and sizes. Macrophages harvested from normal healthy mice found to me more adhesive as evident from the formation of cytoplasmic extensions from all sides of the cells (Fig. 1A). When macrophages harvested from DL-bearing mice, heterogeneity in shape and sizes were observed as in normal macrophages. However, sizes of macrophages were found to be larger than the normal macrophages with densely stained cytoplasm (Fig. 1B). Further, shape was observed to be more roundish in the macrophages harvested from DL-bearing mice compared to the normal macrophages. However, both the macrophages were found to contain eccentric remiform or fusiform nucleuswith one or two distinct nucleoli. The cytoplasm was found to contain fine granules and multiple pink-purple, large azurophilic granules. The cytoplasmic borders were observed to be irregularly serrated.

When macrophages harvested from normal healthy and DL-bearing mice were observed in Scanning Electron microscope, more prominent membrane ruffles and surface blebs were seen in the macrophages harvested from normal healthy mice (Fig. 1C) compared to the macrophages harvested from DL-bearing host (Fig. 1D).

3.2. Effect of DL cell conditioned medium on the morphology of normal macrophages

When NMO was incubated in DL cell conditioned medium (DLCM) at 37°C in an atmosphere of 5% CO_2 and 95% air 1 hr, it was found that the cytoplasmic extensions in the form of lamellipodia reduced. Unlikely to the macrophages stimulated with PMA for 1 hr, the macrophage incubated in DLCM were seen more heterogeneous group of cells. The cytoplasm was clearer with scattered large granules. The N:C ration was found to be reduced compared to the macrophages incubated in complete culture medium and stimulated with PMA for 1 hr (Fig. 2A and 2B).

When the macrophages incubated in DLCM under aforementioned culture condition for 1 hr were analysed under SEM, the membrane ruffling or microvilli was found to be sparse in comparison to the NMO incubated in complete culture medium under culture condition and stimulated with PMA for 1 hr. The surface texture was smooth and acquired completelyroundish shape suggesting that DLCM resulted in reduced adhesion of the NMO to the substratum (Fig. 2C and 2D).

3.3. Effect of PMA on the morphology of normal peritoneal macrophages

When NMO was incubated in complete culture medium at 37°C in an atmosphere of 5% CO_2 and 95% air and stimulated with PMA for 1 hr, it was found that the cytoplasmic extensions in the form of lamellipodia increased on all sides (Fig. 3A and 3B). Cytoplasm was observed to be densely stained indicating that PMA stimulation resulted in the accumulation of cytoplasmic granules. However, macrophages were found to be more uniform in comparison to unstimulated macrophages. Further, N:C ration appeared to be increased after PMA stimulation of normal macrophages.

When the macrophages incubated in complete culture medium under aforementioned culture condition and stimulated with PMA for 1 hr were analysed under SEM, the membrane ruffling or microvilli was found to be prominently expressed with numerous cytoplasmic extensions or lamellipodia on all sides. The macrophages were properly adhered to the substratum (Fig. 3C and 3D).

3.4. Effect of DL cell coincubation on the morphology of normal macrophages

When macrophages were co-incubated with DL cells complete culture medium at 37°C in an atmosphere of 5% CO_2 and 95% air 1 hr, results were found to be corresponding as observed in the macrophages incubated in DLCM for 1 hr at respective culture condition. It was found that the cytoplasmic extensions in the form of lamellipodia reduced. Unlikely to the macrophages stimulated with PMA for 1 hr, the macrophages were seen more heterogeneous corresponding to the DLCM incubated macrophages. However, cytoplasm was found to be densely stained with fine granules all over the cytoplasm unlikely to the DLCM incubated macrophages where sparsely

distributed large granules were observed. The N:C ration was found to be increased compared to the macrophages incubated in DLCM and comparable to the macrophages incubated in complete culture medium and stimulated with PMA for 1 hr (Fig. 4A,B).

When the macrophages co-incubated with DL cells in complete culture medium under aforementioned culture condition for 1 hr were analysed under SEM, the membrane ruffling or microvilli was found to be sparse in comparison to the NMO incubated in complete culture medium under culture condition and stimulated with PMA for 1 hr. The surface texture was smooth and acquired completely roundish shape corresponding to the shape of macrophages incubated in DLCM suggesting that similar to DLCM, DL cell co-incubation also resulted in reduced adhesion of the NMO to the substratum (Fig. 4C, D).

3.5. Effect of DL cell conditioned medium on the adhesion of peritoneal macrophages

Further, to assess the effect of DLCM on the adhesion of peritoneal macrophages harvested from normal healthy mice, adhesion assay was performed as described in Materials and methods.It was observed that the adhesion of peritoneal macrophages increased with the increase in time of incubation. Those macrophages which were incubated in uncoated wells showed strong adhesion compared to those macrophages which were incubated in albumin coated wells (Fig. 5A). Further, the stimulation of macrophages with PMA resulted in increased adhesion (Fig. 5B) while the incubation of macrophages in DLCM resulted in reduced adhesion in comparison to the macrophages incubated in incomplete culture medium RPMI1640 with or without PMA stimulation (Fig. 5C). Though, PMA stimulation to the macrophages incubated in DLCM resulted in increased adhesion of peritoneal macrophages but comparatively lower than the macrophages incubated in RPMI1640 culture medium with PMA stimulation (Fig. 5D).

3.6. Effect of DL cell conditioned medium on MNGC formation in macrophages

To assess the effect of DLCM on the formation of multinucleated giant cells, the normal macrophages (NMO) were incubated in complete culture medium or DL cell conditioned medium (DLCM) with or without PMA for different time periods of 6 to 48 hrs and number of MNGC per view field was counted. It was observed that MNGC formation was detected not earlier than 24 hrs of incubation of NMO in DLCM. Further incubation resulted in progressive increase in the appearance of MNGC in the NMO incubated in DLCM. Maximal appearance of MNGC was found at 48 hrs of incubation of macrophages in DLCM. PMA stimulation slightly reduced the formation of MNGC in DLCM culture. On contrary, when NMO were incubated in complete culture medium only, no MNGC was observed at any time period of incubation. However, interestingly, when NMO were incubated in complete culture medium and stimulated with PMA, MNGC was observed as early as 24 hrs of incubation and found maximal at 48 hrs of incubation but significantly lower than the

NMO incubated in DLCM with or without PMA stimulation (Fig. 6A). Correspondingly, the number of nuclei per giant cell increased with the increase in time of incubation. Maximum number of nuclei per giant cell was found at 48 hrs of incubation and onward in DLCM. The PMA stimulation resulted in reduced number of nuclei per giant cell in the macrophages incubated in DLCM while increased number of nuclei per giant cell in the macrophages incubated in complete culture medium compared to that of macrophages incubated in complete culture medium only (Fig. 6B). Corresponding to both observations, the percent fusion of macrophages increased with the increase in time of incubation. Maximum percent fusion of macrophages was found at 48 hrs of incubation and onward in DLCM. The PMA stimulation resulted in reduced number of percent fusion in the macrophages incubated in DLCM while increased number of percent fusion in the macrophages incubated in complete culture medium compared to that of macrophages incubated in complete culture medium only (Fig. 6C).

3.7. Effect of cytokine neutralization on DL-induced MNGC formation in macrophages

As it is evident that tumor-microenvironment constitutes different cytokines and growth factors. Therefore, further experiment was carried out to show the effect of cytokine neutralization on the fusion of macrophages in *in vitro* experimental settings. Result showed that there was significant reduction in the formation of MNGC when anti-IFN-γ antibody or anti-IL-4 antibody were added to the DL cell conditioned medium. When both anti-IFN-γ and anti-IL-4 were added to the DL cell conditioned medium, more pronounced reduction in the macrophage-macrophage homotypic fusion and subsequent MNGC formation was observed (Fig. 7) which indicates that these cytokines are at least partly involved in inducing multinucleate giant cell formation in macrophages in the tumor-bearing host.

4. Discussion

Peritoneal macrophages harvested from DL-bearing host ultra-structurally differ from those of normal healthy mice. Macrophages harvested from DL bearing are larger in size, have less cytoplasmic extensions, smooth surface texture and low adherence to the substratum resulting in roundish shape while macrophages harvested from normal healthy mice comparatively smaller in size, have prominent surface ruffles, properly adhered to the substratum resulting in oval and flattened shape. With these distinct morphological features of normal peritoneal resident macrophages and tumor-associated macrophages led us to easily characterize both the phenotype of macrophages. However, both type of macrophage show heterogeneity in their population.

We have observed that normal resident macrophages incubated in complete culture medium RPMI 1640 supplemented with FBS and stimulated with PMA exhibit more uniform in shape and sizes, increased cytoplasmic granularity specifically accumulation of densely stained fine granules and volume-to-surface ration, increased

N:C ration and highly enhanced membrane ruffles and cytoplasmic extensions in the form of lamellipodia from all sides. The incubation of macrophage in DL cell conditioned medium not only results in heterogeneity in shape and sizes, increased cytoplasmic granularity specifically accumulation of multiple pink-purple, large azurophilicgranules, increase in volume-to-surface ration, increased N:C ration, but also reducedmembrane ruffles. Cytoplasmic extensions in the form of lamellipodia are lacking leading roundish shape compared to the oval or flattened shape of PMA activated normal macrophages. Corresponding results from normal macrophages co-incubated with DL cells in complete culture medium RPMI 1640 supplemented with FBS suggest that the tumor cells are as well involved in the alteration of external morphology of macrophages which is corroborated with the studies performed by Sokol et al., 1990 in haematological malignancy [20] and in other tumor models [26]. However, mechanism(s) that triggers such immense morphological alterations in the macrophages co-incubated with DL cells remains unclear. It might be possible that DL cell-derived soluble factors might be one factor that causes morphological changes in the macrophages. Direct DL cells and macrophage cell-to-cell contact might also be possible mechanism of DL cell-induced morphological alterations.

Tumor development not only induces alteration in morphological features of macrophages, but it also modulates the adhesion of macrophages to the substratum. The macrophages incubated with DLCM conditioned medium show prominent reduction of adhesion to both albumin coated and uncoated substratum which is parallel to the previous observation performed in several other tumor models including breast cancer and tumor metastasis [27, 28]. On contrary, PMA stimulation significantly augments the adhesion of macrophages to both albumin coated and uncoated substratum. However, PMA stimulation to the macrophages incubated in DL cell conditioned medium results in comparatively lower adherence to the substratum in comparison to the macrophages incubated in incomplete culture medium RPMI1640. Increased adherence of PMA activated macrophages indicates that PMA may activate signal cascade in the macrophages that induces the expression of adhesion molecules such as CD54 or ICAM-1, LFA-1 etcon the cell surface of macrophages [29]. It has been enhanced adhesion is resulting from activation of focal adhesion kinases (FAK) in the macrophages [30]. Phorbolmyristate acetate (PMA) and many cytokines such as tumor necrosis factor (TNF)-α, interferon (IFN)-γ, TNF-α with IFN-γ, and EGF enhance macrophage adherence probably by activating FAK in the macrophages [29, 31, 32]. However, exposure to IL-1 [31] GM-CSF [33] results in inhibition of adherence by the loss of expression of adhesion molecules on the macrophage surface.

It has been demonstrated that macrophage co-incubated with tumor cells for longer period results in the formation of multinucleate giant cell formation (MNGC), however mechanism governing the tumor-mediated formation of MNGC is lacking. Advance stage of Dalton's lymphomais found to induce macrophage-macrophage homotypic fusion and MNGC formation (unpublished observation). The macrophages incubated in DL cell conditioned medium results in time dependent increase in fusion

of macrophages when cultured for longer period with increasing level of multinucleation, but when macrophages are incubated in DL cell conditioned medium added with anti-IL-4 or anti-IFN-γ, there is decrease in percent fusion of macrophages. When DL cell conditioned medium is supplied with both anti-IL-4 and anti-IFN-γ, significantly higher reduction in percent fusion of macrophages is observed which is corroborative to the previous studies suggesting that IL-4 and GM-CSF [34-36] and even inflammatory cytokine IFN-γ [37, 38] treatment to peritoneal macrophages lead to the formation of MNGC when incubated for longer periods. The tumor microenvironment has been known to contain immunoregulatory cytokines IL-4, IL-8, IL-10, IL-13 and TGF-β, and growth factors such as M-CSF, G-CSF and GM-CSF [39-41]. Therefore, it can be assumed that the formation of MNGC in the macrophages is due to the presence of tumor-derived immunoregulatory cytokines and growth factors in the DL cell conditioned medium. The reduced formation of MNGC in the macrophages incubated in DLCM which are supplied with anti-IFN-γ and/or anti-IL-4 antibodies further supports this assumption.

5. Conclusion

It may be concluded from the above study that the incubation of macrophages in DL cell conditioned medium results in significant alterations in external morphology characterized by reduction in membrane ruffles and cytoplasmic extensions in the form of lamellipodia, large azurophilic cytoplasmic granules and increase in volume-to-surface ration and N:C ration. DL cell condition medium leads to the changes in shape from oval or flattened to roundish due to loss of adherence to the substratum. Further, DL cell condition medium leads to the formation of MNGC in time dependent manner when incubated for longer period with simultaneous increase in multinucleation.

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Figure Legend

Fig. 1.Morphological characterization of normal peritoneal macrophages and tumor-associated macrophages. Macrophages were harvested from both normal healthy and DL-bearing mice, and adherence purified. Adherent cells were characterized by non-specific esterase staining and subjected to double staining as well as SEM analysis as described in Materials and methods. **(A)** Shows the double staining image of peritoneal macrophages harvested from normal healthy mice (NMO), **(B)** Shows the double staining image of peritoneal macrophages harvested from DL-bearing mice (TAMs), **(C)** Shows the SEM image of peritoneal macrophages harvested from normal healthy mice (NMO), and **(D)** Shows the SEM image of peritoneal macrophages harvested from DL-bearing mice (TAMs) at the magnification indicated at the bottom of images. Images in inset in lower panels indicate images of the same as shown at higher magnification (as indicate at the bottom). The images are representative of six or seven experiments done in duplicate.

Fig. 2. Effect of DL cell conditioned medium on the morphology of normal peritoneal macrophages. Macrophages were harvested from normal healthy mice, adherence purified and characterized. Macrophages were incubated in DL cell conditioned medium (DLCM) for 24 hrs and external morphology were observed by subjecting them to double staining and SEM analysis as described in Materials and methods. **(A)** Shows the double staining image at 63x magnification of normal macrophages stimulated with PMA, **(B)** Shows the double staining image at 100x magnification of normal macrophages stimulated with PMA, **(C)** Shows the SEM image of at 1,000x magnification of normal macrophages stimulated with PMA, and **(D)** Shows the SEM image of at 10,000x magnification of normal macrophages stimulated with PMA. The double staining images are representative of six or seven experiments done in duplicate while SEM images are representative of four experiments done in triplicate.

Fig. 3.Effect of PMA on the morphology of normal peritoneal macrophages. Macrophages were harvested from normal healthy mice, adherence purified and characterized. Macrophages were incubated in complete culture medium and stimulated with PMA for 24 hrs, and external morphology were observed by subjecting them to double staining and SEM analysis as described in Materials and methods. **(A)** Shows the double staining image at 63x magnification of normal macrophages stimulated with PMA, **(B)** Shows the double staining image at 100x magnification of normal macrophages stimulated with PMA, **(C)** Shows the SEM image of at 1,000x magnification of normal macrophages stimulated with PMA, and **(D)** Shows the SEM image of at 10,000x magnification of normal macrophages stimulated with PMA. The double staining images are representative of six or seven experiments done in duplicate while SEM images are representative of four experiments done in triplicate.

Fig. 4.Effect of DL cell coincubation on the morphology of normal peritoneal macrophages. Macrophages were harvested from normal healthy mice, adherence purified and characterized. Macrophages were co-incubated with $\times 10^2$ DL cells in complete culture medium

for 24 hrs and external morphology were observed by subjecting them to double staining and SEM analysis as described in Materials and methods. **(A)** Shows the double staining image at 63x magnification of normal macrophages stimulated with PMA, **(B)** Shows the double staining image at 100x magnification of normal macrophages stimulated with PMA, **(C)** Shows the SEM image of at 1,000x magnification of normal macrophages stimulated with PMA, and **(D)** Shows the SEM image of at 10,000x magnification of normal macrophages stimulated with PMA. The double staining images are representative of six or seven experiments done in duplicate while SEM images are representative of four experiments done in triplicate.

Fig. 5.Effect of DLCM on adhesion of macrophages. Macrophages were harvested from normal healthy mice and adherence purified. Macrophages were incubated in complete culture medium with or without PMA stimulation or incubated in DLCM with or without PMA stimulation for different time periods as indicated and the percent adhesion was calculated described in Materials and methods. **(A)** Shows the percent adhesion of macrophages incubated in incomplete culture medium RPMI1640 only, **(B)** shows the percent adhesion of macrophages incubated in RPMI1640 medium and stimulated with PMA, **(C)** shows the percent adhesion of macrophages incubated in DLCM only, and (D) shows the percent adhesion of macrophages incubated in DLCM and stimulated with PMA with ( ) or without ( ) albumin coating. The values represent mean ± SE_M of three independent experiments performed in triplicate and taken as significant at $p<0.05$.

Fig. 6.Effect of DLCM in MNGC formation in the macrophages. Macrophages were harvested from normal healthy mice and adherence purified. Macrophages were incubated in complete culture medium with or without PMA stimulation or incubated in DLCM with or without PMA stimulation for different time periods as indicated and the formation of multinucleated giant cell (MNGC) was observed and counted as described in Materials and methods. **(A)** Shows the number of giant cells, **(B)** shows the maximum number of nuclei per giant cells, and **(C)** shows the percent fusion of macrophages. The values represent mean ± SE_M of three independent experiments performed in triplicate and taken as significant at $p<0.05$. Sign * indicates significantly higher value to their respective group of macrophages.

Fig. 7.Effect of cytokine neutralization on DLCM-induced MNGC formation in the macrophages. Macrophages were harvested from normal healthy mice and adherence purified. Macrophages were incubated in complete culture medium or DLCM with or without anti-IL-4 or anti-IFN-γ or anti-IL-4 + anti-IFN-γ for 48 hrs and the formation of multinucleated giant cell (MNGC) was observed and counted as described in Materials and methods. The values represent mean ± SE_M of three independent experiments performed in triplicate and taken as significant at $p<0.05$. Sign *indicates significantly lower percent fusion to their respective group of macrophages.

Figure – 1

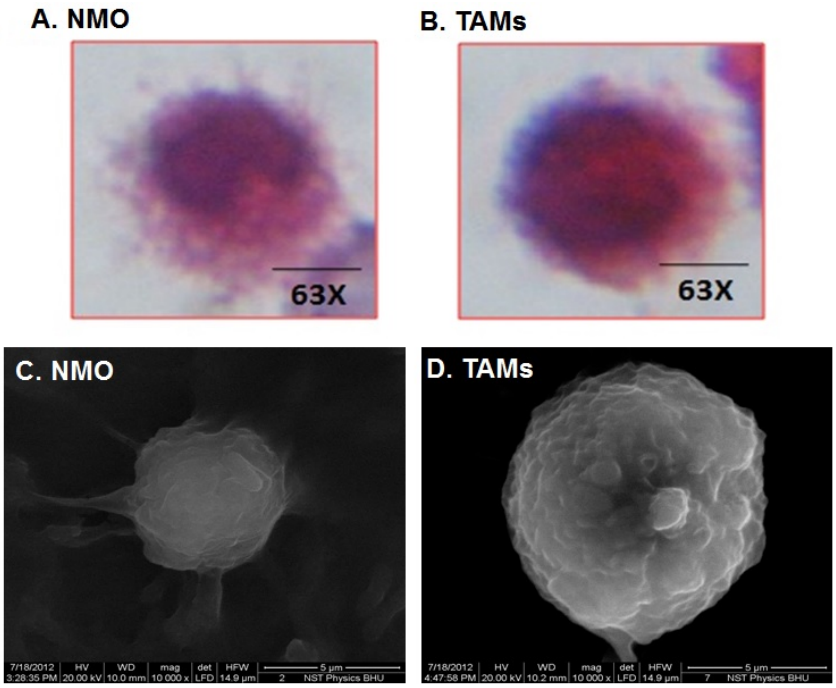


Figure – 2

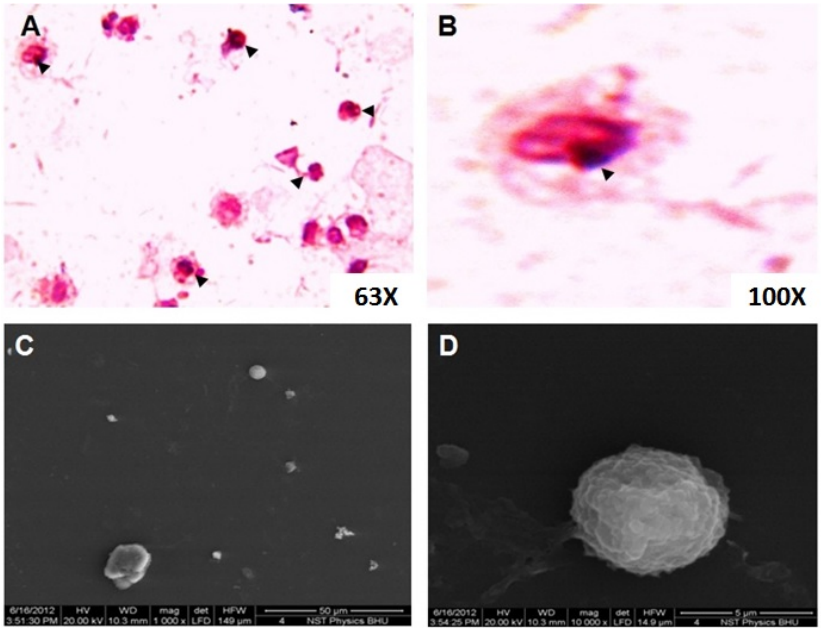


Figure – 3

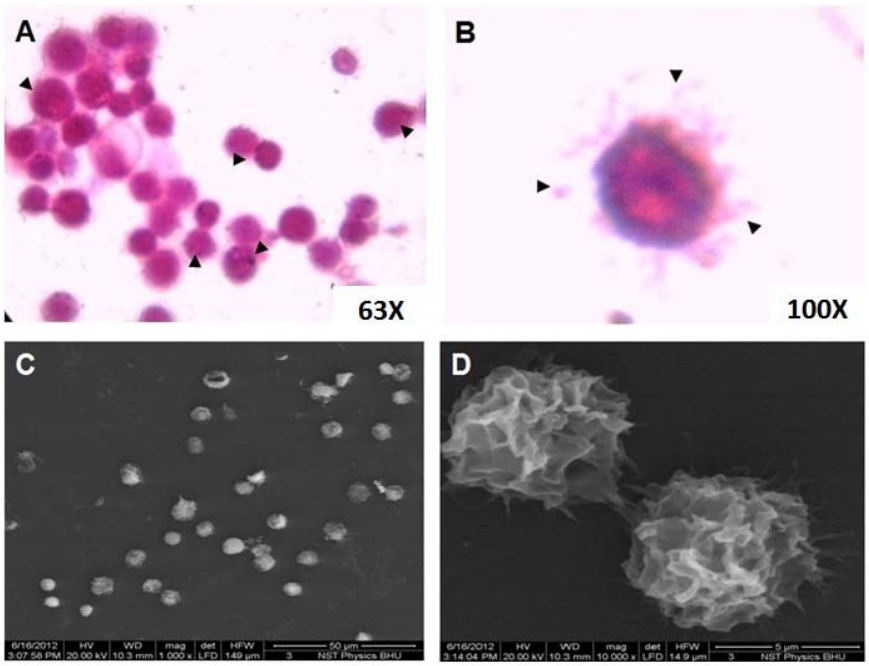


Figure – 4

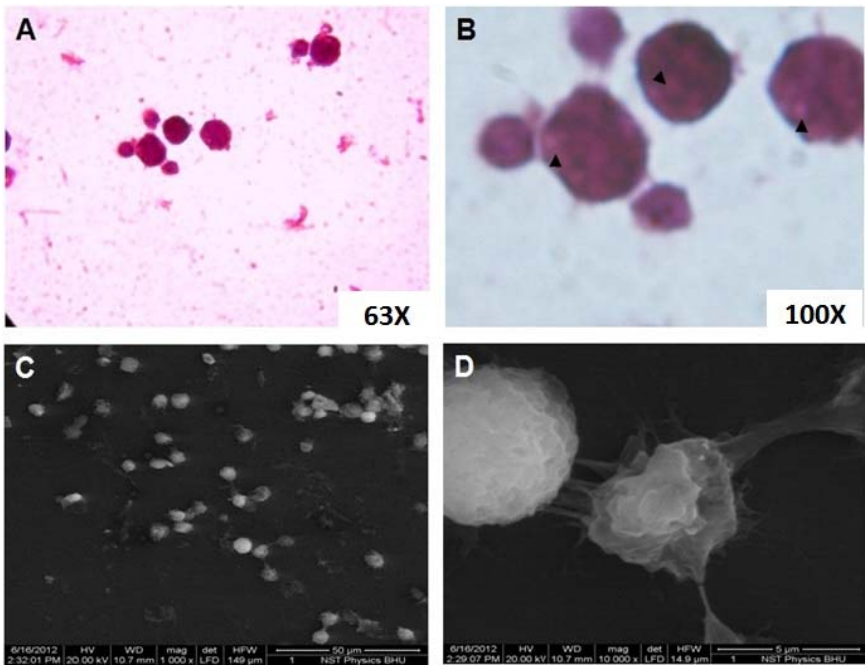


Figure – 5

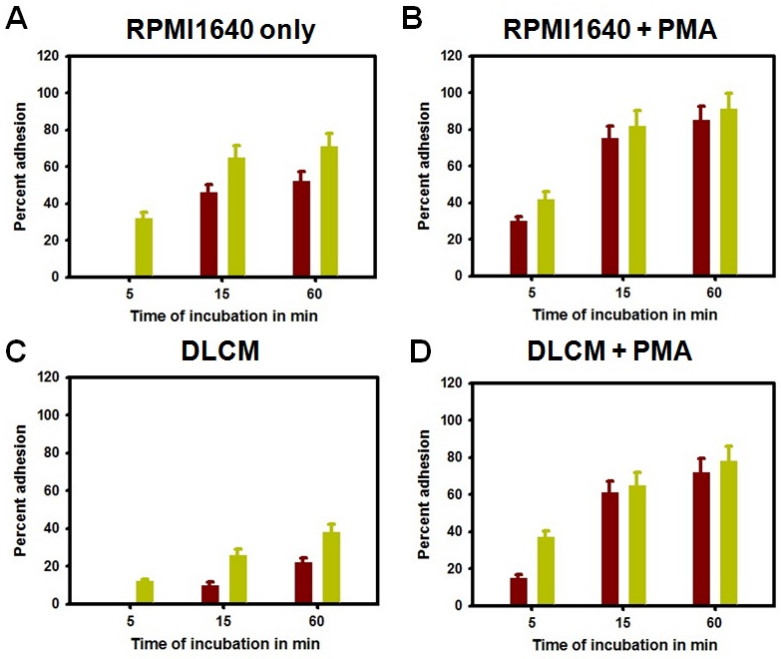


Figure – 6

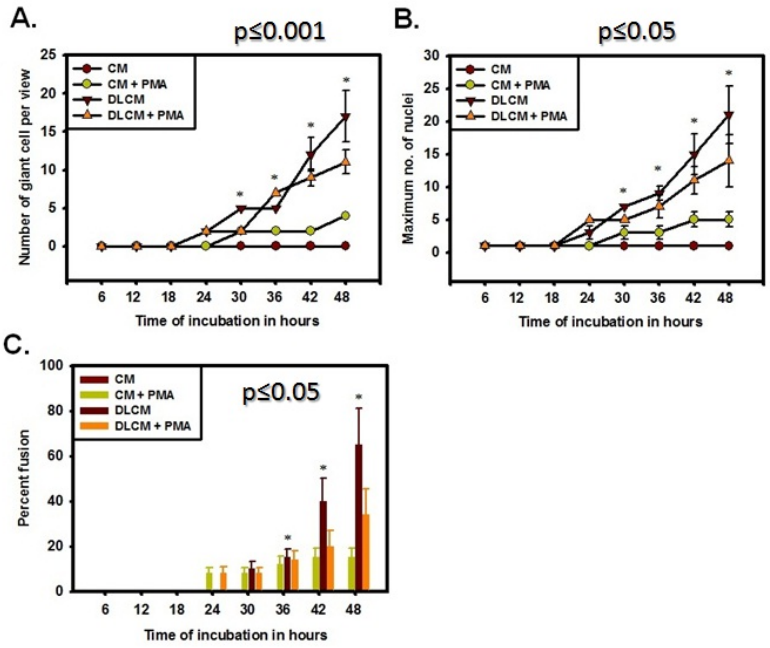
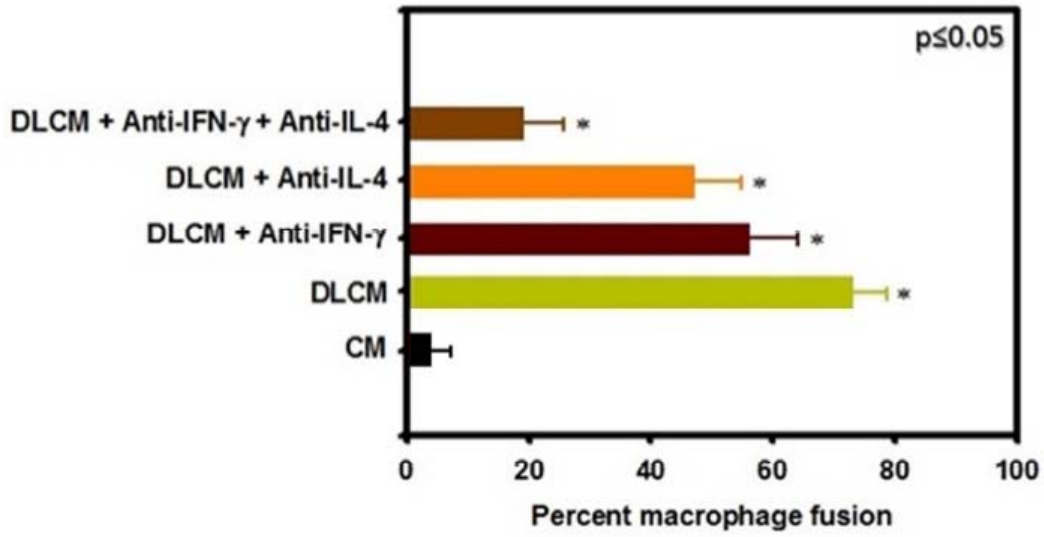


Figure – 7 :


p≤0.05
DLCM + Anti-IFN-γ + Anti-IL-4
DLCM + Anti-IL-4
DLCM + Anti-IFN-γ
DLCM
CM
*
*
*
*
0
20
40
60
80
100
Percent macrophage fusion