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The Effect of Chemokine Receptor Antagonists on the Migration of Tumour Associated Macrophages

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Abstract

The macrophages especially the macrophages associated with the tumour development are the main focus for the experimental purpose. During the experiment, the RAW 264.7 mouse macrophage cell linewascultured in aseptic conditions and then polarized into the M1 and M2 phenotypes. The cytokines such as IL-4, IFN-γ, and IL-4 were used for the induction of polarization. The expected cellular markers of M2 such as CD204 and CD206 has been shown by Western blot analysis. The M1 and M2 phenotypes were evaluated for phagocyticactivity, M2 phenotype being recorded with highest relative phagocytic activity. The chemo tactic movement of RAW264.7 macrophages kept in co-cultre with B16F10 mouse melanoma cells was similar to the transmigration obtained by RANTES (CCL5) alone. The α-RANTES antibody and the CCR2 antagonist significantly reduced transmigration of macrophages in co-culture with B16F10 cells.

Keywords: Macrophages, Melanoma cells, INF-γ, Transcription 1, Myeloid leukemia

Introduction

In mammals, macrophages are well known in all mammalian tissues in which they demonstrate vast efficient and anatomical differentiation. In common, macrophages can be classified by mononuclear phagocytic system, which contain their vastly phagocytic cells and their originators from bone marrow. In addition that the other categorization concerning macrophages is belong to the inflammation, as well as the binary classification. The activated macrophages are considered into the derivative M1 and M2 macrophages, are the major focus of concern [1].

Tumor development

Responses concerning infections, cancer initiation, macrophages can produce inflammatory mediators like INF-γ, TNF-α and IL-6, which can likely involve in other inflammation initiation, which promote the tumor progress. TNF-α is the major mediator in the generation of carcinogenic environment. However, TAM (tumor-associated macrophages) can change the immunogenic environment to immunosuppressive surroundings that initiate tumor progression and malignancy. IL-4 based differentiation has the major role in tumor development [1].

M1 mancrophages are liable for the phagocytic activity. M1 macrophages are stimulated classically for the immune responces. The m1 macrophase removes the pathogens (the foreign particles). The M2 macrophages stimulate the reparations and tumour growth and the the assistance INF-γ and IL-4 are specifically involved in the induction of the polarization of the M1 and M2 phenotypes. These mediators are responsible for the synthesis of the TNF-α (tumour necrosis factor-alpha). M2 macrophages are the alternatively activated [2].

M1 stimulation can be classified according to their effectiveness to initiate the inflammatory responses and markers. INF-γ is the major cytokine mediator connected with M1 stimulation and major product of the Th1 cells. The structure belonging to the INF-γ receptor is the INFGR-1 and INFGR-2 chains. The receptor initiates the activity of STAT1 (signal transducers and activators of transcription 1) and interferon regulatory factors (IRF) such as IRF-1 and IRF-8. The activity of LPS in the M1/M2 stimulation is the difference from INF-γ on gene expression. In human beings the lack of the INF-γ receptor results in the immunodeficiency syndrome [2].

The M2 stimulation can be carried out by IL-4 mediator. Glucocorticoids are also be involved by the M2 category, but they have a various types of stimulus. Glucocorticoides – stimulated monocytes are mediated by NFkB shows the expression of complement characters like subunit A (C1QA), thrombospondin 1, IL-10, has the major impact on which binding towards the IL-10R1 and IL-10R2 takes place, those are the subunits of the receptor which increases the auto-phosphorylation, ends into the initiation of transcription of STAT 3 and cytokine expression. Gene alteration in the M-CSF receptor genes leads to the myeloid leukemia [3] (Table 1).

Review of literature on activity of macrophages

- Macrophages (mononuclear phagocytes) have very important role towards homeostasis of innate and adaptive immune systems [1].
- Macrophages have also impact on tissue remodeling and wound healing. Macrophages can be isolated and characterized by various methods like in vitro culture techniques.
- The biology of macrophages has been concerned with the development of many kind of tumor progession [1].

MARKERS	
MOUSE	
M1 macrophase	CXC9, CXCL10, CXCL11, NOS2
M2 macrophase	Mrc1, tgm2, Fi221, YM112, Arg I
HUMAN	
M1macrophase	MRC1, TGM2, CD23, CCL22
M2macrophase	CD64, ID0, SOCS1, CXCL10

Table 1: Selectivity of receptors in M1 and M2 macrophages [3].

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- TAM (tumor associated macrophages) can alter the immune responses towards promoting tumour growth [1].
- According to the literature, CD 36 have the migration efficacy of mouse as well as umanmacrophages, for that the oxidized LDL had been used for the evaluation purpose [4].
- The cell membrane proteins (markers) on macrophages can be analyzed by proteomic techniques such as LC-MS/MS, SDS-PAGE electrophoresis. The examples of proteins to be analyzed are CXCL243, CXCL98, CXCL107a, CCX107b,CXCL36, CCX97, CXC205, CXC206, CXC180, CXC191, CXC300, CXC45, and CXC29 [5,6].

CD (Cluster differentiation) receptors

This kind of receptors are used for the identification of the cell molecules for the investigation purpose. The cluster differentiation proteins are used for the cell adhesion and the best targets for the macrophase cells. The CD proteins can provide the information regarding specific antibodies proposed by the different laboratories around the world (Table 2).

Stem cells	CD 34+, CD 31-, CD 117
Leukocyte	CD 45+
Granulocyte	CD 45+, CD 114+
Monocyte	CD45+, CD11a
T-lymphocytes	CD 45 +
T-helper cell	CD 3+
T-regulatory cell	CD4, CD25
Cytotoxic T cell	CD3+
B lymphocytes	CD45+
Thrombocyte	CD61+
Natural Killer cells	CD 3-

Table 2: Types of cell CD markers [6].

MRC-I (macrophase mannose receptor 1)	C-type mannose receptor 1 C-type lectin domain family 13 membrane D CLEC 13D, CD206, MMR
MRC2- macrophase mannose receptor 2	C-type mannose receptor 2, urokinase type plasminogen activator receptor-associated protein, CD280

Table 3: The main histocompatibilty complex molecules are of mannose receptors (Tachado et al.)

Progression of tumor	
Mechanism	Mediator (s)
Enhancement of growth	Growth factors (EGF, PDGF, TNF-b) Cytokines(IL-6, TNF-α) L-arginine derived polyamine
Enhanced Angiogenesis	Through production of various cytokines (GM-CSF, TGF-α, TGF-β, IL-1, IL6,IL8) Prostanoids (Procogulant activity)
Invasion and Dissemination	Cytokines (TNF-α, IL1) Lytic enzymes (Metaloproteases and plasminogen activator)
Immunosuppression	Prostanoids (PGE2), cytokines(IL-10), and other mediators (TNF-b)
Downregulation of the tumor cells	
Direct cellular cytotoxicity	Cell to cell contact
Antibody-dependent celluar cytotoxicity	Fc-receptor(CD16)
Secretory products (cytotoxic/ cytostatic)	Ecosanoids (PGs, LTS) Cytokines (IL-1, TNF-α) Free redicals (R10, NO)
Macrophase induces apoptosis	TNF-α, IL-10, R-10, NO

Table 4: TAMS and Tumor Interaction (progression and downregulation) [4].

CD3 receptor

This kind of receptor is composed of protein complex consisting of CD3γ, CD3δ and CD3ε chains. The otelixizumab, the monoclonal antibody is responsible for the blockade of the CD3 receptor [7].

CD4 receptor

This kind of protein is a glycoprotein that is found on the surface of the T-helper cells, monocytes and dendritic cells. It is fallen in the class of immunoglobuline super family. CD4 is a main CD-receptor with a good communication with antigen presenting cells. CD4 receptor may act by interaction with MHC class II molecules on the surface of the antigen presenting cells [8].

Mannose receptor

This kind of receptor has a major role in the innate and adaptive immune systems. These receptors are frequently recycled in between the plasma membrane and endosomal compartments in a clathrin-dependent manner. The mannose receptor is the kind of the type 1 transmembrane protein. It has an extracellular N-terminus and the intracellular C-terminus. The N-terminus cystien-rich domain, fibrinolectintype-II repeat domain and C-type carbohydrate recognition domains are responsible for the ligand binding and responsible for phagocytosis activity. The mannose receptor are responsible for the interaction of the pathogens and then directs it to the lysosomes for degradation (Table 3) [9].

TAMS (Tumor associated macrophages) and cancer cells

Mononuclear phagocytes have a number of growth factors, for example platelet derived growth factor, epidermal growth factor and transforming growth factor (TGF-b) (Table 4) [4].

Chemokine receptors

The chemokine receptor are the 7-transmembrane structural proteins, and are the members of the G-protien couple receptors.

Family of chemokine receptors:

- CXC receptors
- CC chemokine receptors
- C Chemokine receptor
- CX3C receptor

Mechanism of action of chemokine receptor

Transduction of signals: When the chemokine ligand binds to the chemokine receptor, exchange of GDP takes place in the place GTP, Gβ unit of the GPCR initiates the activity of (PLC) phospholipase C. The activation of the phospholipase forms the cleavage of phosphoinositol (4,5) biphosphate (PIP2). The PIP2 forms the two messengers (IP3) inositol triphosphate and diacylglycerol (DAG). The DAG initiates the activity of PKC (phosphokinase C). IP3 rises the intracellular stores of calcium ions. The ions triggers the MAPkinase pathway, activating the specific cellular mechanisms such as chemotaxis etc [10,11].

CXCR3 receptor

It is the Gαi protein complex associated with the GPCR. It has two variants CXCR3-A and CXCR3B. CXCR3-A binds to the

CXCL9, CXCL10 and CXC11. CXCR-3 are the major receptors on cell membranes of T lymphocytes and NK cells.

Chemokine receptor antagonists

In insulin resistance, the monocyte chemoattractant protein-1 named (chemokine ligand 2) (CCL 2) plays a vital role for chemokine receptor 2. The RS504393, the antagonist of chemokine receptor CCR2 have an impact on the migration of the tissue macrophages.

Materials and Methods

Cell cultration

The 264.7 mouse melanoma cells were obtained and collected with the help of the aseptic culture medium. The Dulbecco modified minimal essential medium was used for the cultration of the cells. The sterile phosphate buffer saline was used for the removation of the cells from the culture plates. The cells were incubated for 37°C in 5% CO_2. The cells were then centrifuged and processed adequately.

Splitting of the RAW 264.7 mouse macrophase cell lines

- The splitting could be done by use of Dulbeco's liquid medium.
- The grown culture might be washed with filtered sterile PBS solution.
- Then addition of 1 ml of cell cuture had been done to each cell culture dishes.
- Liquid culture medium of 9 ml was added to each dishes (culture plates).
- Washing should be discarded.
- Then after washing, 1 ml of the trypsin solution was added for resuspending the macrophase cell lines.
- The incubation for macrophase cell lines should be done at 37°C for 5 minutes.
- Moreover, the observation regarding the macrophase detachment could be done by using the light microscopy by tapping the dishes gently.

Polarization of the RAW 264.7 mouse macrophase cell lines

M1 stimulation: The macrophase cell lines can be converted into the phenotypic M1 cells by adding the INF-γ and LPS to the grown cell lines

Calculation

Formula: Ca*Va = Cb*Vb

Where, Va = Volume required from the stock solution in µl Ca = concentration of stock solution (0.1 mg/ml)

Vb = Total volume of culture required (10 ml)

Cb = The final concentration

Therefore,

Mediator	Stock concentration(mg/ml)	Volume (µl) required for 10 ml of cell culture
IFN-γ	0.1	2
LPS	1	1
IL4	0.1	2

Table 5: Calculated values for the chemical mediators for the mouse macrophase 267.4 cell lines.

Va = 0.1*10/1 (mg*ml*ml/mg*ml)

Va = 2 µl

The same calculation could be done for the LPS. The required volume is 1 µl from the 1 mg/ml stock solution.

M2 stimulation: The macrophase cell lines could be converted into the phenotypic M2 cells by adding IL4 (0.2 µg) to the grown cell culture.

The calculate volume for the IL4 is 2 µl from 0.1 mg/ml stock (Table 5).

Lysis of the Cells

The media should be removed from the plates. The plates must be washed with the 5-6 ml of the ice cold PBS and the plates should be placed on the ice. Then the lysis of the M1 and M2 cell line should be took placed by adding the 500 µl of ice cold lysis buffer on the cells of M1 and M2. The cells must be scrapedoff with a plastic scraper. The lysed solution of the cells should be pippeted out in the eppendorf tube. The cells could be of centrifuged at about 13,000 RPM at about 5 minutes. The supernatant must be cleared into the newly labeled eppendorf tubes. This solution should be stored at -80°C fo further use.

The composition of the lysis buffer:

20 mM tris HCl pH 7.5, 150 mM of NaCl, 10% of glycerol, 1% of triton X 100 and 2 mM of EDTA. The protease inhibitors were added to complete the lysis buffer.

Phagocytosis assay

Reagents

Phalloidin: Rhodamine-phalloidin is the highly- specific F-actin binding fluorescence labelled protein. Phalloidin selectively binds to the F-actin. Rhodamine (fluorescence dye) has the emission wavelength of 565 nm.

DAPI: This kind of fluorescence stain binds strongly to the A-T region of the genomic DNA. Bound DAPI-DNA complex has an absorbance at 358 nm and them emits the light at 461 nm.

Triton X 100: The permeabilization of the cell membrane was done by the use of the chemicals such as triton-X. This is necessary for the better access to transmembrane and intracellular molecules. The disadvantage of using this chemical is because of the non-selectivity towards the proteins and the lipids.

FBS: Fetal bovin serum serves in this case as blocking agent. Phosphate buffer saline: Washing buffer.

DMEM (Dulbecco modified minimal essential medium): It contains the essential vitamins, aminoacids and glucose. Additionally, it contains iron and phenolred. This culture media is suitable for the growth of the mammalian cells.

4% PFA: Fixative.

Direct staining of the actin cytoskeleton of the cell line of the mouse macrophages (M0, M1 M2)

Direct staining is required for the visualization purpose. Fluorescence-conjugated Phalloidin was used for the visualization of the actin cytoskeleton in the mouse macrophages.

Procedure

- The medium had to be removed from the chamber slide.

- The cells should be washed with the 2 ml of the wash buffer (0.5% FBS in PBS).
- Fixation might be done with the help of 0.5 ml of PFA for 15 min at the room temperature.
- 4% PFA had to be removed and had to be collected in the waste tube.
- Again the cells should be washed with the wash buffer (3*0.5 ml)
- The cells was then permeabilized with the use of 0.5 ml of 0.1% Triton–X of about 15 minutes at the room temperature.
- Washings of the cells was done with the wash buffer.
- Incubation of the cells with the solution of phalloidin (phalloidin-TRITC 1: 1000 dilution).
- DAPI solution added to the slide for visualization purpose.

Phagocytic assay

Flourescence conjugated yeast particles (Zymosan) were added into the cell cultures of M0, M1 and M2 cell types. The cells were incubated on ice for 2 h.

The number of phagosomes per cell should be counted and the calculation has to be done for the further evaluation. Phagocytosis by macrophages is proposed under the category of the innate immune response. The mannose receptors are invoved in this kind of activity.

BCA (bicinchoninic assay) The concentration of protein can be measured by color change of the protein solution and subsequent absorbance measurement.

BCA reaction includes the followings

Bincinchoninic acid, sodium carbonate, sodium bicarbonate, sodium tartarate, cupric sulphatepeta hydrate.

The BCA reaction depends on the two reactions: 1) the reduction of Ca^{+2} to Ca^{+} by proteins (including peptides bonds). The concentration of Ca^{+} ions is directly propostional to the amount of the protein present in the solution. The bicinchoninic acid forms the chelate with Ca+ ion, which is purple colored product that absorbs the light at a wavelength of 562 nm. The absorbance can be quantified by the UV spectrometer.

Western blot technique (WBT)

Reagents for the western blot analysis

1. 10 X transfer buffer: it includes the 121.1 g of tris base, 576 g glucose and make up to the volume 4 L with ddH_2O.
2. 1 X transfer buffer includes the 700 ml of ddH_2O, 100 ml of 10 X transfer, 200 ml of methanol and make up to the volume with 700 ml of ddH_2O.
3. 20 X TBS includes the 193.6 g tris base, 640 g of NaCl and make up to the volume of 3.2 L with ddH_2O. Adjustment of pH 7.6 should be done with concentrated HCl. Then make uo to the volume of 4 L with ddH_2O.
4. 20 X TBST: it can be prepared by using the tween 20 of 2 ml to the 100 ml of 20 X TBS solution. TBST is the washing buffer.
5. 5% milk blocking solution: it can be made by using the 25 g semi-skimmed milk powder and then make up to the volume of 500 ml by using 500 ml of 1 X TBST.

SDS PAGE electrophoresis

Components: The buffer commonly used for the SDS-PAGE gel electrophoresis is the tris buffer, which is useful for the adjustment of the pH. The counter ions are used to alter the mobility of the proteins depending on their nature. Acrylamide is used for the polymerization and hydrolysis of the gel. The sodium dodecyl suphate is used for the denaturation of the protein to the linear state. This covers the protein with negative charge for the electrophoretic mobility. Ammonium persulphate is useful to initiate the gel formation. TEMED (N,N,N', N'tetramethylethylenediamine), equalize the free redicals and then provide the reasonable polymerization reaction.

Preparation of the resolving gel: Resolving gel can be prepared by mixing the solutions of Acrylamide, 1.5 M tris/ HCl (pH8.8), ddH_2O and 20% SDS. The TEMED and APS should be added at last. After that the gel mixture should be quickly transferred to the gel assembly (equipped with the glass plates and pressure cams: it must tested for the leakage.). The surface of the gel should be covered with the n-butanol for making the surface even. The gel must be kept for 15- 30 minutes the polymerization.

Preparation of the stacking gel: Meanwhile the stacking gel had to be prepared by mixing the acrylamide, 0.5 M tris/HCl (pH6.6), ddH_2O, 20% SDS. The TEMED and the SDS was added to the last with the similar fashion. The gel mixture should be transfer on the top layer of the resolving gel.

Method for SDS PAGE electrophoresis (Table 6).

Order of loading of protein samples on the developed gel

1) PAGE ruler of 5 µl
2) Mo (non polarizedmacrophages)
3) M1 (polarized macrophages)
4) M2 (polarized macrophages)

Preparation of the samples

1) For the Mo (non polarizedmacrophages) 4.96 µl of the lysate cell solution should be mixed with 11.04 µl of buffer and 4 µl of loading buffer.
2) For the M1 macrophages (267.4 mouse macrophase cell line): 6.23 µl of lysate cell solution was mixed with the 9.77 µl of lysate buffer and 4 µl of the loading buffer.
3) For the M2 macrophages: 4 µl of the lysate cell solution should be mixed with the 12 µl of the lysate buffer and the 4 µl of the loading buffer.

All the samples should be preheated at about 100°C for about 2 minutes for inactivation of the proteins.

Tranfer of the proteins from the gel to the membrane

The proteins from the gel can be transferred on the nitrocellulose

Solution	12% resolving gel	4% stacking gel
Acrylamide	590 µl	330 µl
1.5 M tris/HCl (pH 8.8)	650 µl	-
0.5 M tris/HCl (pH 6.6)	-	650 µl
ddH2O	1.24 ml	1.51 ml
SDS	13 µl	13 µl
TEMED	3 µl	3 µl
APS	13 µl	13 µl

Table 6: Componets for the preparation of resolving and stacking gel.

membrane. The proteins can be transfer in to the membrane by using the electrical current. The materials like sponges and gel itself and the membrane can be used for the preparation of the sandwich for the transfer. Then this sandwich should be placed in the chamber containing transfer buffer of pH 7.4 with appropriate volts and electric current supply. The generated electric field can transfer the proteins to the membrane. This sandwich should be kept overnight appropriate transfer.

Procedure

1. Preparation of TBS solution should be done. Then preparation of TBST solution should be done. The membrane should be incubated with 5% of the milk for atleast 1 hour. It is the blocking solution. The total volume was 20 ml.
2. After that, the membrane should be washed with the TBST buffer solution for atleast 3 minutes.
3. The membrane had been incubated with primary antibody in 5% of the milk for atleast1 hour. (mannose receptor specific). (the primary concentration should be higher)
4. Then again, the membrane had been washed with TBST solution for atleast 10 minutes. This washings should be done for three times.
5. Afterwards the membrane must be incubated with the secondary antibody targeted towards the species of the first antibody in the TBST solution for atleast 50 minutes.
6. The secondary antibody should have the enzymes such as peroxidase to provide the chemiluminescence.

Detection

ECL western blot: The enhanced chemiluminescence method is useful for the detection of the subtract which is present at very low concentration. The presence of the peroxide enzyme initiates the oxidation process of compounds such as luminal. This oxidation reaction produces the 3-aminophthalate from the luminal through various intermediates. Moreover, the existence of various compounds, the intensity of the emitted light could be increased upto the 1000-times. This gives the detection of light very sensitive. Thus, the sensitivity of the method can be increased up to 10-100 levels. Appropriate filters can be used to optimize the detection limit. When the radioactive labeled enzyme substrates exposed to the X-ray light through the use of the X-ray film, the dark areas are created on the film which are to be correlated with protein bands. (which are present on the western blot membrane).

Chemotactic transmigration assay: The cells (B16F10 mouse melanoma cell lines) had to be placed in the lower chamber. The RAW 267.4 cell lines of mouse macrophages had to be placed on the membrane of the upper chamber. On the first instance, the RANTES was placed in the lower chamber as chemoattractant. After 24 h of incubation at 37°C, the membrane had to be removed and on its lower surface the transmigrated macrophages had to be counted. The cells became visible under fluorescence microscope after DAPI staining (Figure 1).

The similar process of cell counting was done for the B16F10 mouse melanoma cell lines, the co-culture of macrophages with B16F10 mouse melanoma cell lines + α-RANTES antibody againstCCL5,and the co-culture of macrophages with B16F10 mouse melanoma cell lines + CCR2 antagonist. The percentage of the relative cell migrationwascalculated according to the cell counting.

Results

Phagocytosis: (Figure 2, Graph 1 and Table 7).

BCA assay: According to the graph, for BCA assay, the equation was found to be Y = 0.003X + 0.025, r2 = 0.995.

The observed absorbance for the samples were found to be 0.038, 0.043 and 0.072.

For, M0 = 0.038, M1 = 0.043 and M2 = 0.072.

Therefore, Y = mx + C, so, X = (Y-C)/M.

From the equation, the volume of M0 = 4.96 µl, M1 = 0.042 µl and M2 = 0.037 µl.

These are the required volume for preparation for the western blot analysis from the stock solutions of the respected cell lysate solution (Graph 2 and Table 8).

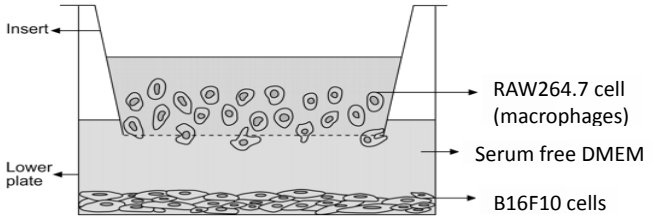


Figure 1: Chamber for the evaluation of the chemotactic activity of the above mentioned call lines. (Modified after Tsai et al. [11].

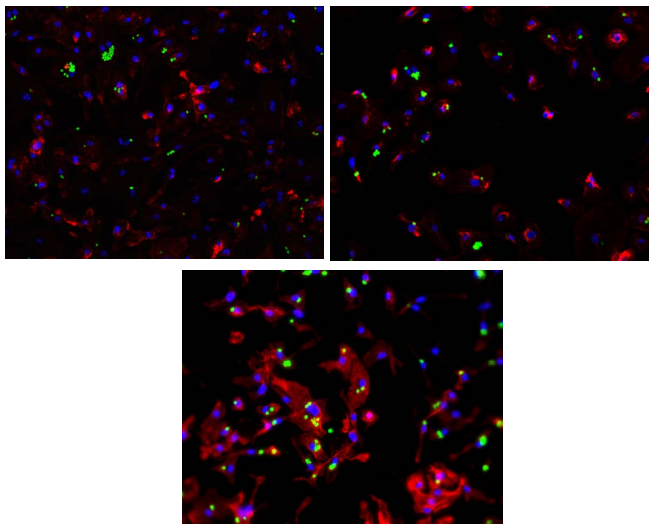


Figure 2: *Courtesy of Dr Annamaria Gal:* (a) Florescence microscopic image of Phagosomes(green) with the M0 unpolarisedmacrophase.s (b) Florescence microscopic image of M1 polarised macrophages, the blue dots indicate the centromer part of the nucleus (c) Florescence microscopic image of M2 polarised macrophages.

(a) The blue dots represent the DAPI stain of the nuclei, the red area is for the F-actin stain and the green dots represent the fluorescence stain of the yeast phagosomes *(courtesy of the Dr. Annamaria Gal)*.

(b) The blue dots represent the DAPI stain of the nuclei, the red area is for the F-actin sain and the green dots represent the fluorescence stain of the yeast phagosomes *(courtesy of the Dr. Annamaria Gal)*.

(c) The blue dots represent the DAPI stain of the nuclei, the red area is for the F-actin sain and the green dots represent the fluorescence stain of the yeast phagosomes (courtesy of the Dr. Annamaria Gal).

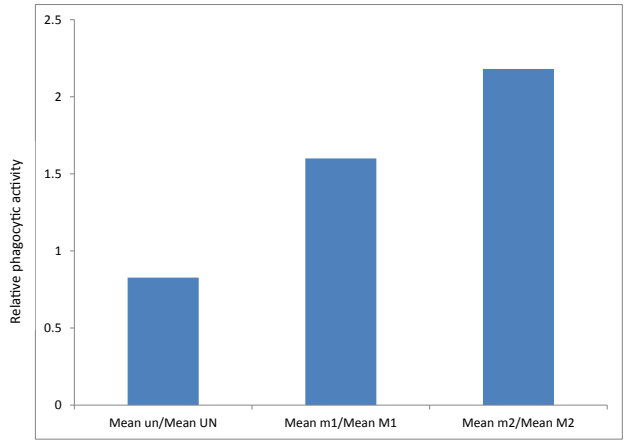
Western blot technique

The western blotting is the analytical tool for the measurement of the specific proteins(CD206, CD204 and β-actin) in the cell lysate. It includes the gel electrophoresis technique for the separation of the proteins. Then the separated proteins are then transferred into the membrane. The membrane is made of nitrocellulose. The transferred are projected towards the antibodies to stain the proteins. This technique has been used for the different disciplines (Figure 3).

Colorimetric detection: the proteins are to be detected depending on the color development, and quantified by the desitometric analysis.

Chemiluminescent detection: on this detection, the substrateis exposed to the reporter on the secondary antibody. The CCD cameras have been used to the western blot. Desitometry has been used for the quantification purpose and further analysis like molecular detection.

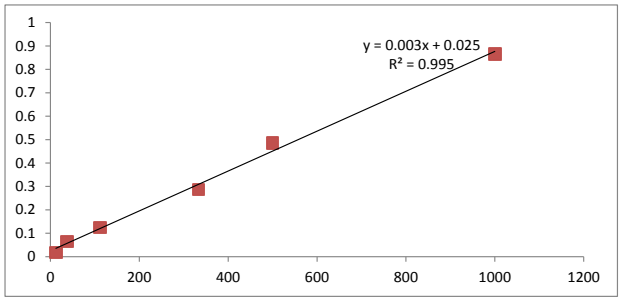
Flourescence detection: the labeled fluorescence probe can be excited by the light and the emission from the probe can be detected by the sensor such as CCD cameras. The use of appropriate filters has been used for the quantification purpose. This detection method used for the quantification and molecular weight analysis.



Graph 1: Relative phagocytic activity of the macrophasis (Blue Area).

No of phagocytised particles	Unpolarized	m1	m2
	50	126	84
	77	50	83
	74	52	80
AVG	67	76	82.33333333
SD	14.79864859	43.31281566	2.081665999
Total no. of cells	UN	M1	M2
	69	64	40
	86	40	39
	88	38	35
AVG	81	47.33333333	38
SD	10.44030651	14.46835628	2.645751311
Ratios	Mean un/Mean UN	Mean m1/Mean M1	Mean m^2/Mean M^2
	0.827	1.6	2.18

Table 7: Phagocytic assay result for the macrophages.



Graph 2: BCA method results.

Concentration(µg/ml)	Optical density
12.35	0.019
37.04	0.065
111.1	0.126
333.3	0.288
500	0.485
1000	0.866

Table 8: Absorbance readings for the standards.

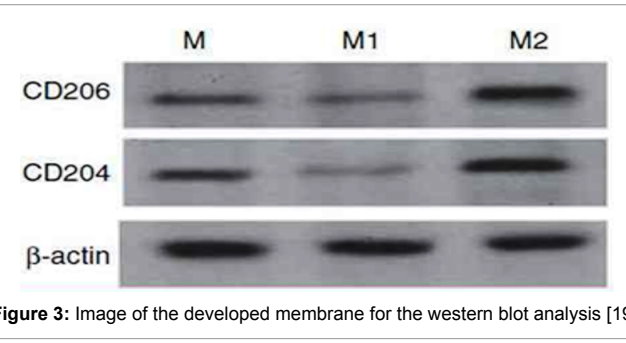


Figure 3: Image of the developed membrane for the western blot analysis [19].

Chemotactic assay (Graph 3 and Table 9).

Discussion

According to the observation of the phagocytosis assay the mean m1/Mean M1 is in between

Where, m1 = number of phagosome in M1 cells,

M1 = total number of M1 cells observed.

The observed Mean un/ Mean UN and Mean m2/ Mean M2,

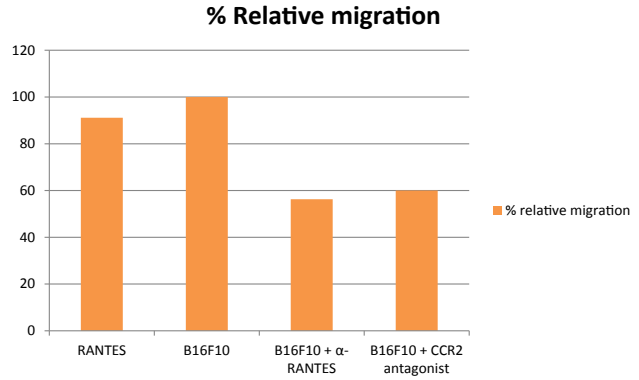
Where mean m2 = number of phagosomes in M2 cells and mean M2 = total number of M2 cells.

Mean un = total number of phagosomes in unpolarised cells,

Mean UN = total number of unpolarised cells.

According to the graph, the unpolarised cells have the less relative phagocytosis activity having the standard deviation of 14.79 for number of phagosomes and 10.44 from the total number of cells. The M1 macrophages have the in-between relative phagocytosis activity having the standard deviation of 43.31 for number of phagosomes and 14.46 for total number of cells. The M2 macrophages have the highest activity of SD of 2.64 for total number of cells and 2.081 for number of phagosomes.

The ratios for unpolarised cells is Mean un/Mean UN = 0.827, for



Graph 3: Relative migration efficacy for the different sorts of above mentioned cell lines. (The first bar is for the positive control (chemokine RANTES), with that the migration of macrophages had been recorded on lower membrane of the migration chamber. The second bar is for the B16F10 mouse mellnoma cell lines, the migrated mecrophases were of 100% as relative the others. The third bar is for co culture B16F10 mouse melanoma cells and α-RANTES antibody, the relative migration of the macrophages were deceased to about 56 %. The relative migration of macrophages towards the co culture B16F10 mouse melanoma cell line and CCR2 antagonist on the lower membrane of chamber was 60 %).

Chemoattractant	% relative migration	SEM	T-test
RANTES	91.14	5.41	0.04435
B16F10	100	7.65	
B16F10 + α-RANTES	56.27	4.48	
B16F10 + CCR2 antagonist	59.99	5.57	

Courtesy of Dr Annamaria Gal. The T-test was performed for the finding of the significant difference between the chemoattractants [RANTES,+B16F10 cells] and co cultures (B16F10 with α-RANTES antibody and CCR2 antagonist).

Table 9: Chemotactic assay results.

polarized M1 macrophages. Mean m1/ Mean M1 = 1.6 and for polarized M2 macrophages,

Mean m2/ Mean M2 = 2.18.

Chemokine receptors

Chemokine receptors are composed with G-protien coupled receptors having 7- transmembrane domains. The chemokine receptors are on the surface of the macrophase cell lines. Accorging to the literature, 19 chemokine receptors have been found. They all have the common intracellular and extracellular hydrophilic loops, having the phosphorylation sites.

The mannose receptor has been involved in the stimulation of the murin macrophages by IL-4 and IL-13. They are involved in the high endocytic clearance, increament in the major histocompatibility complex (MHC) class II production. There is an increment in the mannose receptors in response to the IL-4 to IL 10, to the polarization of the macrophages in the M1 and M2 phenotypes. IL-4 stimulate the induction of macrophages fusion and delays the phagocytosis [12-14].

The mannose-6-phosphate receptors are involved in the tumour development. The compound CI-MPR is bound to the mannose-6-phosphate receptor and also to the domain II of the IGF-II. It was established that the mice which is deficient in the CI-MPR, could have the increased level of IGF-II and the enlarged organs. CI-MPR has the ability to alter the level of IGF-II, having the major role in the tumour suppression [15].

Mannose receptor governs the engulfment of the microorganisms and glycoprotiens. CD 63 has the target role in the interlazation of the acidic phagosomes. Mannose receptors are the pattern type of recognition receptor. They are expressed on the cell surface of macrophages. The mannose receptor complexes with the Ags on the bacterial cell surface and the major target for the engulfment. Furthuermore, the MHC- class II is also the complexing agent with the mannose receptor. Mannose receptor complexes with the MHC- class II through the involvement of the IFN-γ.

Western blotting

The bands (Figure 3) were observed if the western blot would developed for the chemilumenescent detection. The membrane had the bands those were very intense in the lane of the M2 phenotype. Acoording to that the mannose receptors are more expressed in M2 phenotype of the macrophages in comparison with the M0 and M0 cell types. Generally, the mannose receptor (CD 206) has the molecular weight of the 175 KDa. The M1 lane has the quite faint band, indicating that the expression of CD 206 is less. The bands regarding to the β- actin region has the similar light intensity for all three bands, indicating that the loading was equal for all samples.

The migration inhibitory factors for the macrophages have the major role in the regulation of the innate immunity. The receptor involved in the release of MIF from white blood cells is CD 74. The glucocorticoids are also the mediators for the secretion of MIF from the white blood cells. The mannose receptors can have the binding affinity through the involvement of the fibronectin type II domain by attaching to the collagen like carbohydrate structures and mediates the activity like engulfment of bacterias and acididcphagosomes [16].

Mannose receptors in the tumour progression

The mannose receptors are also known as the CD206 receptors could have being highly expressed in the tumour associated phenotypes. These phenotypes are M1 and M2 macrophages. The main proteins involved in the tumour development mucins and the residues containing the glycolipids (the GPI sites). The binding of mesothelin like structures depend on the existence of the GPI sites and the mannose receptor itself. The mesothelin is the target ligand for the activation of the mannose receptor. The antibodies towards the 4th domain of the mannose receptor could interfere the binding of the mesothalin to the mannose receptor and the GPI sites itself. These cell processes contribute towards the inhibition of the polarization of tumour associated macrophages. These anti-mannose receptor antibodies are the target focus of concern in the ovarian cancer [17]. The cytokine receptor on the tumour associated macrophages could also be blocked by the nanobodies anti-mannose receptor nanobodies and the anti-cytokine receptor nanobodiescould selectively inhibit the polymerization and phenotypic regulation of the macrophages by blocking the targeted sites of the cytokine receptors [18]. The chemical equilibrium associated with the epithelial- mesenchymal sites is the main focus of concern related to the micro-environmental changes in the biology of the systemic growth of the tumerogenic development and also towards the metastasis. M2-polarised macrophages has thought to be mainly involved in the EMT (epithelial mesenchymal transition) in the pancreatic cancer. The TLR-4 (toll like receptor-4) is expressed more during the chronic inflammation and sustained development of the tumor on the phenotypic macrophase type-II. The chemical mediators such as IL-10, IL-4 have the major focus on the cell line of 264.7 (phenotypic macrophase type II). There is the marked increased in the expression of CD 206 and CD 204 on the cell surface of the macrophages. At the mRNA and protein levels, the existence of the prolonged levels of the vimetin and snail, those are

of the mechenchymal markers. The enzymes such as metalloproteases have thought to be associated in the lysis of proteins in the tumerogenic cell lines of pancreas [19].

Although, the marker such as E-cadherin in the co culturation with the M2-macrophages shows that there is the downregulation of the levels of E-cadherin at the epithelial sites. The techniques like silencing RNA, knockdown techniques, antibodies towards the toll-like receptor 4 and IL-10 have an significant role in the downregulation of E-cadherin levels and increased expression of vimentin as well as snail [19].

The anti-inflammatory activity of macrophages have thought to be involved by the markers like CD 204 and/or CD 163 at the cell membrane of the phenotypic type–II macrophages. There is upregulation of expression of endothelial growth promoting factors due to the high mRNA-protiens (CD 204) levels [19].

Toll like receptor 4: TLR-4 is mainly concerned with the tumerogenesis of many cancer cell lines. The mainly involved pathway for the development of tumerogenesis is the MyD88-dependent inhibition. The activity of MyD88 would likely to increase the amount of metalloproteases (MMPs) in the cancer cell lines/ macrophages phenotypes. This enhanced levels of MMPs has been concerned with the TAMs (M2 polarised cells) [19].

TLR-4 receptor in M2- phenotypic cells is related to the release of IL-10, leads to the tumerogenesis. The connection between the ratios of mRNA/protein (hypoxia inducible transcription factor alpha) and mRNA/protein TLR-4 have been found positive in the metastasis of tumors [19].

Molecular weight of CD 204: 50 KDa,

Molecular weight of β-actin: 15 KDa,

Molecular weight of CD 206: 180 KDa.

Cell migration assay

Mannose receptor has been involved in the infection, inflammation and migration of the macrophages. It is the transmembrane protein. The mice species which are deficient in the mannose receptor have a deficiency in the cell migration. MR- deficient mice has an increased migratory cell types such as bone marrow macrophase phenotypes. The mannose receptor has an molecular weight of the 175 KDa.

CCL5: (RANTES) is the chemokine secreted and expressed by many cell types including the hematopoietic and non-hematopoietic types. CCR5 antagonist can be of very useful for the development of drugs for AIDS disease. The secreted CCL5 have an effect on the migration of the TAMS (tumour associated macrophages). CCL5 (RANTES) receptor targeted antibodies have an significant effect on the migration of the RAW 264.7 cell lines of the mouse macrophages. For that the α-RANTES should be co-cultured with the B16F10 mouse melanoma cell lines. The percentage of relative cell migration was found to be 56.27% [19].

CCR2: It is the chemokine receptor with the molecular weight of 13 KDa. It is secreted by many cells associated with macrophages. This sort of protein can be metabolized by metalloprotien (MMP-12). This chemokine is responsible for the chemotactic movement of the macrophages. The antagonist towards the CCL2 receptor has been associated with the anti-migratory effect on the macrophages. According to the experimentation, the co-cultured CCR2 antagonist with B16F10 mouse melanoma macrophages had the percentage of chemotactic activity was found to be 59.99%, as compared to the 100% chemotactic activity of RAW 267.4 mouse macrophages towards the B16F10 mouse melanoma cell lines [19].

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