Tracking and traversal of monocyte derived Dendritic Cells (MoDCs) in animal model(s)

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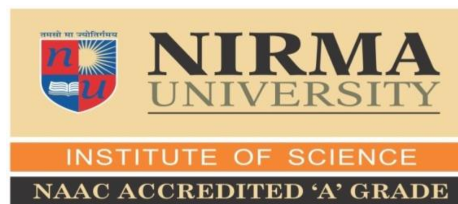
In Partial fulfilment of requirement for
The Degree of

MASTER OF SCIENCE

IN

BIOTECHNOLOGY AND BIOCHEMISTRY



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Abbreviations

DCs: Dendritic Cells

MoDCs: Monocyte Derived Dendritic Cells

MHC: Major histocompatbility complex

IL: Interleukin

Th1: T Helper cells

CD: Cluster of Differentiation

GM-CSF: Granulocyte Macrophage Colony Stimulating factor

DC-SIGN: Dendritic Cell Specific Intercellular adhesion molecule 3 Grabbing Non – integrin

PAMPs: Pathogen associated molecular patterns

JAK/STAT: Janus kinase /Signal Transducer and Activator of Transcription protein

P13K/PKB: Phosphatidylinositol 3-kinases/ Protein kinase B

MAPK/ERK: Mitogen-activated protein kinases/ extracellular signal-regulated kinases

NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells

PBMC: Peripheral blood mononuclear cell

PBS: Phosphate Buffer Saline

RPMI: Roswell Park Memorial Institute medium

CFSE: CarboxyfluorescinSuccinimidyl Ester

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Abstract

To induce a well effective cellular immune response, there must be a good antigen presenting cell which can interconnect adaptive and innate immune system effectively. In this study, we aimed to generate effective monocyte derived dendritic cells from peripheral blood in vitro and track them in mouse models using fluorescence microscopy. To carry out this study, we isolated monocytes from human peripheral blood by Ficoll gradient method. Upon obtaining the monocytes, GM-CSF, a growth stimulating factor and IL 4, a cytokine that inhibits monocyte differentiation into macrophages were added. After 7 days, the immature monocyte derived dendritic cells (MoDCs) were labeled with CFSE tracking dye and administered into immunosuppresed wild type mice. Immunosupression of mice was carried out by delivering clodronate enclosed liposomes into the mice. After 24 hrs, the mice were euthanized, various organs were collected and organ slides were prepared. Thereafter, the slides were analyzed by fluorescence microscopy Also, cells samples were prepared for confirmation of MoDCs through flow cytometry by tagging the cells with characteristic DC markers, CD1c and DC-SIGN respectively. This study explores future cancer treatment and vaccine development (DCs as adjuvant) for various disease and disorders. Tracking the route of *in vitro* generated MoDCs gave us an idea about the target site of the DC vaccine. Results obtained are consistent to what was reported earlier, and deciphered new target sites for DC vaccine such as lungs and kidney. The phenotype of *ex-vivo* generated monocyte derived DCs was confirmed by flow cyotmetry.

Introduction & Background

Dendritic cells are known to be potent antigen presenting cells. DCs are the bridging gap between innate and adaptive immune system by presenting antigen and secreting various co-stimulatory molecules and inflammatory mediators such as cytokines, which activate adaptive immunity. (Lipscomb M.F *et al,2002*).The main biological function of DCs is to process and present antigens. These cells (DCs) have been reportedly shown to generate primary immune response in resting naïve T lymphocytes (*Fong L.et al,2000*).To perform this function, DCs are capable of capturing antigens, processing and presenting on the cell surface through MHC-I&II along with appropriate co-stimulation molecules. (*Fong L.et al,2000*).It plays a central role in tolerance and prevention of autoimmunity, maintenance of B cell functions and recall responses. Also, DCs plays an instrumental role in the establishment of immunological memory. (*Same R.et al, 2001*)

Types of dendritic cell include follicular dendritic cells (FDC),lymphoid dendritic cells (lymphopoiesis), myeloid dendritic cells (monocytopoiesis) (MDC1, MDC2) and plasmacytoid dendritic cells(PDC).The secretion of IL12 by MoDCs stimulates T cell responses, in particular the differentiation of Th1 cells, which produce interferon-γ and other inflammatory cytokines (Norimitsu K et al,2002)

Characteristics of DCs

Dendritic cells contain numerous membrane processes that extend out from the main cells with abundant intracellular structures relating to antigen processing including, lysosomes, endosomes and Birbeck granules of Langerhans cells of the epidermis. The detection of DCs is difficult in absence of a single cell surface marker specifically expressed on the surface of DCs. Therefore, a combination of presence and absence of various surface biomarkers has been used to identify DCs. These include the presence of large amounts of MHC II antigens and the absence of various lineage markers such as CD19 (B cell), CD56 (natural killer cell), CD3 (Tcell), CD14 (monocyte), and CD66b (granulocyte).CD86 is an accessory molecule known to be a marker of early DC maturation, while CD80expressed by mature DCs. Two additional markers of mature DC in humans are CD83 and CMRF-44. CD83 expressed by

activated B cells, and CMRF-44 by monocytes and wandering macrophages and monocytes(*Curtis BM et al,1992*).

Function of dendritic cells

The function of DCs falls into three categories, each of which involve antigen presentation and activation of T cells, inducing and maintaining immune tolerance and follicular DCs appear to work to maintain immune memory in co-ordination with B cells.

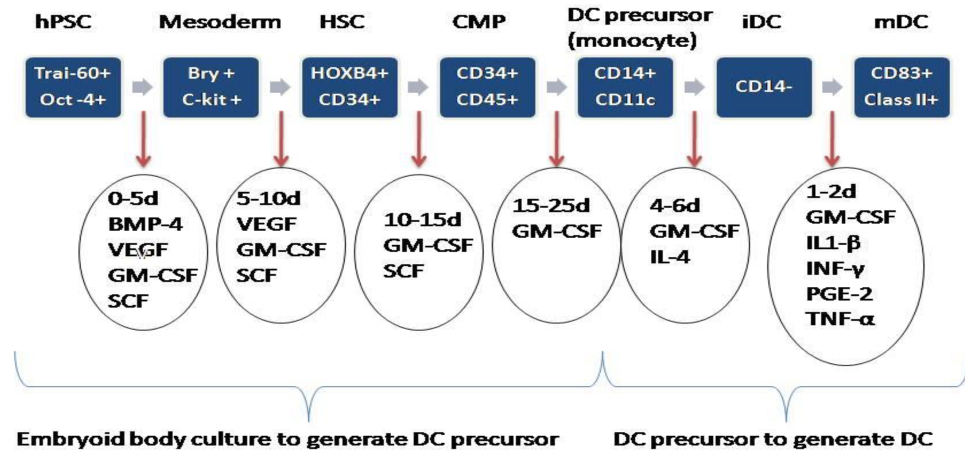
Generation of dendritic cells

DCs can be generated by culturing CD34+ cells in the presence of various cytokines. One approach involves depleting the CD34+ cells of differentiated precursors and then culturing the cells in the presence of GM-CSF and IL-4 ± TNF-α. CD34+ cells can be obtained from bone marrow, cord blood or GM-CSF mobilized peripheral blood. Another approach is to generate DC-like cells by culturing CD14+ monocyte-enriched PBMC. These cultures differentiated into DC phenotype when stimulated with IL-4 and GM-CSF *(McGreal E et al,2005)*.Human clinical trials are ongoing to use DCs to evoke immunity against antigens of breast cancer, lung cancer, melanoma, prostate and renal cell cancers.Morphologically, dendritic cells have a cell body and dendrites arise from it as like in neuron dendrites, and therefore termed dendritic cells. The long fibroblastic morphology of DCs helps them to capture pathogen efficiently. MHC-I and MHC-II are expressed on the surface of DCs. Immature DCs are potently capable in capturing antigen by phagocytosis, and they lose their ability to take up antigen but retain the ability to process and present the antigen to T-cells. DCs recognize Pathogen Associated Molecular Pattern (PAMPs), present on pathogens and are recognized by PRRs(pattern recognize receptor). When a pathogen having PAMPs binds to DCs, it activates DCs to secrete various inflammatory cytokines, which, in turn, helps in the activation of T cells. DCs can be originated from bone marrow CD34+ progenitors or from CD14+ monocytes from peripheral blood. Mainly DCs originate from CD34+ hematopoietic progenitor. DCs which originate from myeloid progenitor are called DC1 or classical DC and expresses Toll like receptor (TLR)-2, 3, 4 and TLR-7. Whereas DC2 originates from lymphoid

progenitor, also called plasmacytoid DCs, expresses only TLR-7 and TLR-9 *(McGreal E et al, 2005)* (*Curtis BM et al, 1992*).

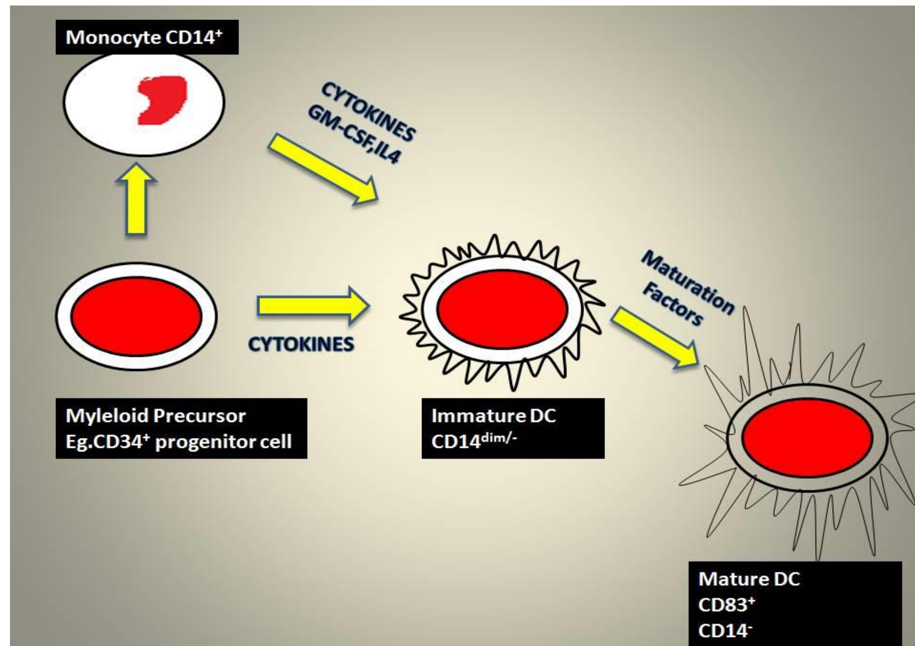
DC-SIGN (CD 209)

DC-SIGN (Dendritic Cell Specific Intercellular adhesion molecule 3 Grabbing Non-integrin) also known as CD209 (Cluster of Differentiation 209) is a protein which in humans is encoded by the *CD209* gene *(McGreal E et al,2005).*DC-SIGN is a C-type lectin receptor present on the surface of macrophages and dendritic cells. DC-SIGN on macrophages recognizes and binds to mannose type molecules, a class of pathogen associated molecular patterns (PAMPs) commonly found in viruses, bacteria and fungi. This binding interaction activates phagocytosis *(Curtis BM et al,1992).* On myeloid and pre-plasmacytoid dendritic cells DC-SIGN facilitates dendritic cell rolling interactions with blood endothelium and activation of CD4+ T cells, as well as recognition of pathogen haptens. In addition, DC-SIGN is reported to trigger innate immunity by modulating toll-like receptors[*den Dunnen J et al,2008*] by employing unknown mechanisms. DC-SIGN along with various other C-type lectins is involved in tumour recognition through dendritic cells. DC-SIGN may act as target molecule to engineer dendritic cell based cancer vaccine [*Aarnoudse CA et al,2006*].



Schematic of the differentiation process of human pluripotent stem cells to dendritic cells

Monocytes circulate in blood, bone marrow, and spleen, and do not proliferate in a steady state (*Auffray C et al, 2009; Swirski FK et al2009*). Monocytes represent immune effector cells, equipped with chemokine receptors and pathogen recognition receptors that mediate migration from blood to tissues during infection. They produce inflammatory cytokines engulf cells and toxic molecules. Also, they differentiate into inflammatory DCs or macrophages during inflammation, and probably less efficiently in the steady state. The migration to deep-seated tissues and differentiation to inflammatory DC and macrophages is determined by the inflammatory milieu and pathogen associated pattern recognition receptors (*Serbina NV et al*, *2008*).



Work flow for *ex-vivo* generation of monocyte-derived Dendritic Cells (MoDCs)

Monocyte derived dendritic cells differ from plasmacytoid dendritic cells by having $CD11c^{+}$,$CD123^{-}$, $BDCA1^{+}$ whereas plasmacytoid DCs have $CD11c^{-}$,$CD123^{+}$, $BDCA2^{+}$ and $BDCA4^{+}$ phenotype(Serbina et al,2008).The dendritic cells are normally differentiated from monocytes to counter infection and thereby induces defense phenomenon, inflammation. These cells defend the host from pathogenic attack and therefore termed as inflammatory DCs (iDCs).

Macrophages are resident phagocytic cells in lymphoid and non-lymphoid tissues, and are involved in establishing steady-state tissue homeostasis via the clearance of apoptotic cells. Macrophages are equipped with a broad range of pathogen recognition receptors that make them efficient at phagocytising and inducing the production of inflammatory cytokines (*Gordon S.et al 2002*).

Classical DCs (cDCs) are specialized antigen-processing and presenting cells, having high phagocytic activity as immature cells and high cytokine producing capacity as mature cells (*BanchereauJ,et al1998*;*Mellman et al 2001*). cDCs are seen in in the peripheral circulation in human and rarely found in in murine blood. cDCs are highly migratory cells that can move from tissues to the T-cell and B-cell zones of lymphoid

organs through afferent lymphatics and high endothelial venules. cDCs regulate T cell responses both in the steady-state as well as to counter infection. They are generally short-lived and replaced by blood-borne precursors (*Liu K,et al, 2009 ;Waskow C, et al2008*).

IL 4 and GM CSF

Interleukin 4 (IL-4) and granulocyte macrophage colony-stimulating factor (GM-CSF) drive dendritic cell differentiation, whereas GM-CSF individually leads to macrophage differentiation (Wiktor-Jedrzejczak W *et al* 1996). GM-CSF was the first cytokine shown to efficiently promote DC development *in vitro* and has been used to induce DC differentiation from human monocytes as well as human and mouse hematopoietic progenitor cells(Kawasaki ES, et al. 1985;Laar, L. et al 2016). The significant increase in DC numbers seen in the spleen and thymus of mice stimulated with GM-CSF or transgenic mice expressing GM-CSF is suggestive of the crucial role played by GM-CSF in DC expansion *in vivo*(Lin H, et al. 2008; McKenna HJ, 2000).The four principle signaling modules activated by GM-CSF; JAK2/STAT5, PI3K/PKB, MEK/ERK, and canonical NF-κB have invariable and overlapping functions in the regulation of GM-CSF–induced DC differentiation and development. Also, these cascades form an integrated network with the activity of one pathway also affecting others.

Introduction & Background

Clodronicacid or clodronatedisodium is a first generation (non-nitrogenous) bisphosphonate. Dichloromethylenediphosphonate can be used for temporary elimination of macrophages in various organs when delivered through lipid vesicles, liposomes. No comparable effect on the macrophages of the spleen was seen with free dichloromethylenediphosphonate or in the case of empty liposomes (NicoVanRooijen et al 2002).Depleted macrophages are replaced by new ones recruited from the bone marrow after various periods of time. In the liver, new Kupffer cells reappear after 5 days, and repopulation of the liver with Kupffer cells is complete after 2 weeks. In the spleen, red pulp macrophages, marginal metallophilic

macrophages, and marginal zone macrophages reappear after1 week, 3 weeks, and 2 months, respectively *(Nico van Rooijenet al, 2003).*

In the present study, 47 million PBMCs were obtained from 40 ml of human peripheral blood. The monocytes were selected from PBMCs by conventional adherence technique. PBMCs were allowed to stand for 2-3 hr of incubation of PBMCs in 5% humidified CO_2 incubator, and selected monocytes were then cultured in the presence of 100ng/ml GM-CSF and 25ng/ml IL-4.The medium as well as the growth factors were replenished each and every other day for the differentiation of monocytes intoMoDCs.5-6 days after incubation; cells were characterized with respect to their phenotype by flow cytometer. The characterized MoDCs were labelled with CFSE and injected (3 million) into wild type C57BL/6mice.The *in vitro* signal sustainability/durability of CFSE was detected at different time points(6hr-72hr)under fluorescence microscope. Upon euthinization of mice, organs were collected, slides were prepared thereafter and were analyzed under fluorescence microscope *(Olympus BX61*).

Objectives

OBJECTIVE-I Isolation of CD14+ monocytes from human peripheral blood

OBJECTIVE-II Ex vivo generation of monocyte derived dendritic cells

OBJECTIVE-III Tracking of human blood cells in animal model(s)

Methods

Isolation of PBMCs from human peripheral blood

40mL of blood was withdrawn from four different donors by using 10mL syringes, and then transferred to heparinised vacutainers. The blood was diluted with PBS (without Ca^{++}/Mg^{++})in 1:1 ratio (40mL blood+40mL PBS).The diluted blood was layered on 10mL lymphosep™ (MP Bio), in four different 50mL falcon tube. All the four tubes were centrifuged at 400g for 40min at room temperature; the deceleration was zero during this process. After 40min the PBMCs made a white layer (buffy coat) just above the lymphosep™, and the layer was harvested by pipette without disturbing the lymphosep layer; Lymphosep is toxic to cells and may affect cell viability. The buffy coat was collected in a single 50mL tube and centrifuged at 200xg for 10min at room temperature three times in physiological PBS. The pellet was then re-suspended in RPMI and cells were counted by haemocytometer. The viability was assessed by Trypan Blue exclusion assay. The PBMC yield and viability will see a decrease when Ficoll/lymphosep is mixed with blood.

Assessment of cell viability

After isolation of PBMCs, total cell number and viability was determined by trypan blue staining by counting the number of cells which have taken up dye using haemocytometer. Trypan Blue is one of several stains recommended for the use in dye exclusion procedures for viable cell counting. This method is based on the principle that live (viable) cells do not take up certain dyes, whereas dead (non-viable) cells do because of alternation in the conformation of plasma membrane. Trypan Blue has a greater affinity for serum proteins to that seen with cellular proteins. If the background is too dark, cells must be pelleted and re-suspended in protein-free medium or buffer prior to counting.10µL cell suspension was taken from a stock volume of 1mL in RPMI 1640 and reconstituted with 10µL of Trypan Blue and 80µL of PBS and allowed to stand for 5min.If cells are exposed to Trypan Blue for a long time the viable as well as dead cells will uptake the dye.10µL of the cell suspension was transferred to the space between coverslip and hemocytometer. The four corner chambers were counted under microscope, and the cell density was calculated. The

cells were counted on top and left touching middle line of the perimeter of each square and cells touching the middle line at bottom and right sides were not counted. Each square of the haemocytometer, represents a total volume of 0.1 mm^3 or 10^{-4} cm^3. Since 1 cm^3 is equivalent to approximately 1 ml, the subsequent cell concentration per ml (and the total number of cells) will be determined using the following calculations.

CELLS/ml = the average count per square ×dilution factor × 10^4

TOTAL CELLS = cells/ml × the original volume of fluid from which cell sample was removed.

CELL VIABILITY (%) = total viable cells (unstained) ÷ total cells (stained and unstained) × 100.

Isolation and differentiation CD14$^+$ monocytes to generate monocyte derived DCs

Approximately 20 million of PBMCs were seeded in a T75mm^2 culture flask in 10mL RPMI 1640 medium containing 10% FCS, 1% HEPES, 1% Pen-strep and 1% glutamine and incubated for 2 hours in CO_2 incubator at 37°C with appropriate humidity. PBMCs consist of various cell types such as lymphocytes, monocytes, macrophages, neutrophils, eosinophils, basophils etc, out of which CD14$^+$ monocytes have the ability to adhere to plastic surfaces due to high expression of β2-intigrin family, monocytes consists of 10%-30% of total PBMCs. Therefore, after 2-3 hour of incubation the population of cells which are adhered are monocytes**.** After 3 hour the non-adhere cells were washed out and the adhered cells were harvested in 7mL RPMI-1640 with 100ng/mL GM-CSF and 25ng/mL IL-4 in a T75 mm^2 culture flask**.** On each and other day medium supplemented with GM-CSF and IL-4 was replenished for5-6 days**.**5-6 after cultivation, the adhered cells are isolated by trypsinisation, stained with FITC conjugated CD1c and CD209 antibodies, and acquired the samples on flow cytometer (BD EACs Canto II) to characterized the phenotype of MoDCs (FlowJO v. 10). Also**,** cells were stained with CFSE and administered to mice for tracking.

***In vitro* assessment of sustainability of CFSE (staining of PBMCs and MoDCs)**

CFSE (Carboxy Fluorescin Succinimidyl Ester) is a fluorescent dye which binds covalently to the lysine residues of cell protein of a cell and the intensity goes down during cell division. Further, this dye is commonly used for T-cell proliferation assay**.** PBMCs in a six well plate were harvested after staining with CFSE (1million each well)**.**The smears were drawn after 6hr, 24hr, 48hr, 72hr, 84hr and 96hr of incubation and observed under fluorescence microscopy to determine signal durability.

CFSE staining

Total of $13x10^6$PBMCs were reconstituted in RPMI-1640 medium.10µM CFSE was prepared in 3mL pre-warmed RPMI-1640 from 10mM of stock solution in PBS. The dye was not added directly to the cells as dye being dense will not stain cells uniformly. Therefore $13x10^6$cells were made upto certain volume in one tube and CFSE dye made in another tube with pre-warmed complete RPMI medium. The suspension of both tubes was mixed together to achieving the 10µMworking concentration of dye. CFSE dye is photosensitive therefore exposure to the light is avoided to refrain the degradation of dye during CFSE staining. Cells were incubated at 37^0C and 5% CO_2 for 10 min during CFSE staining in CO_2 incubator. The staining is quenched by addition of two volumes of chilled complete RPMI media. The cells were pellet down at 1,000 RPM for 7 min. Pellet was re-suspended in the complete RPMI medium and spin as earlier and wash it two times. The pellet was taken on a slide and fixed using 4% paraformaldehyde for 10 min and let it dry. After fixation the cells were protected by a coverslip, with DPX mounting agent then stored until it is images are acquired under fluorescence microscope.

Surface antibody staining of MoDCs for phenotypic characterization

MoDCs were trypsinized and were collected 0.5-1 million per mL.1 million cells were reconstituted in 300µL of RPMI-1640 and distributed in three separate 1.5mL centrifuged tube.5µL of anti-human CD209 mAb and 5µL of anti-human CD1c mAb were added to two separate centrifuge tubes containing 100µL of cell suspension, and the third tube is used as an experimental control. All three tubes were incubated for 20-30min in dark.1mL of washing buffer was added to each tube and centrifuged for

5min at 200g two times with wash buffer(PBS + 1%FBS + 1% sodium azide).The supernatant was aspirated from all the tubes and reconstituted with 400µL of wash buffer, and was then acquired over the flow cytometer.

Immunofluorescence assay (IFA)

3 millions of CFSE labelled cells were injected to wild type mice C57BL/6 through intravenous route following lateral tail vein. After 24hr, 48hr and 72hr mice were euthanized and different organs (liver, lymph node, kidney, lungs, spleen, and heart) were collected. Organs were minced on slide to get sufficient number of cells and let it dry. The minced cells on slides were fixed with 4% paraformaldehyde, and washed 2-3 times with PBS and let it dried. The fixed cells were protected by using coverslip with mounting reagent DPX**.** The slides were observed under fluorescence microscope using PERD, Ahmedabad facility.

Preparation of host

The excessively recruited cells of monocyte-macrophages lineage play a crucial role in clearing injected cells. Therefore, innate immune effecters are controlled by the treatment of clodronate-loaded liposomes intraperitoneal route of administration on every 2-4 days interval for a week. Once the host is controlled for non-adaptive immune response, *ex-vivo* generated MoDCs were administered to mice.

EXPERIMENT-1	**EXPERIMENT-2**	**EXPERIMENT-3**
Three wild type mice+ Injected with CFSE labelled DC 2.4 (murine DC cells, 3 million each) ↓ Euthanized at 24h, 48h and 72h and smears were drawn.	One wild type mice+ Injected with 3 million of CFSE labelled MoDCs ↓ Euthanized after 24h and smears were drawn.	One clodronate-liposome injected mice+ Injected with 3 million of CFSE labelled MoDCs ↓ Euthanized after 24h and smears were drawn.

Formulation and characterization of clodronate-loaded liposome

The clodronate loaded liposome were formulated by disodium clodronatetetrahydrate (TCI chemical Pvt. Ltd, MW=288.85) was used along with soya phosphatidyl choline and cholesterol. 75mg of soya PC and 11mg cholesterol were dissolved in 20mL of dilution of methanol and chloroform (1:1) in a round bottom flask. The organic phase was removed by low vacuum rotary evaporator at 60-65^{0}C. The lipid film was dissolved in 10mL chloroform and was removed by the vacuum evaporator. For clodronate encapsulation,1mL of drug stock solution (0.6 M clodronate solution) was dissolved in 10 mL of PBS and was added to the lipid film and rotated for 15-20 min and milky solution was collected. The milky solution was maintained at room temperature for 2 hours, and sonicated for 3 min at room temperature. The solution was then maintained for another 2 hours to allow liposome swelling. It was filtered through 0.2µm syringe filter and 200µL of this solution was used to be injected to mice

RESULTS

GM-CSF and IL-4 derived MoDCs

PBMCs were isolated from fresh human peripheral blood (40 mL). After isolation of 47 million PBMCs, these cells were incubated for 3 hours in humidified CO_2 incubator, and non-adhered cells were discarded and adhered cells ($CD14^+$ monocytes) were cultured with 100ng/mL GM-CSF (*Invitrogen*) and 25ng/mL IL-4 (*Invitrogen*) in complete RPMI-1640 medium for 6-7 days. After 2 days of culture, these adhered cells showed alternation in morphology and on 6th day the cells represented dendrites like processes.

PBMCs count by haemocytometer

Quadrant	Live cells	Dead cells	Total cells
1	240	6	246
2	275	9	284
3	237	7	244
4	224	16	240
Avg. cells	**244**	**9.5**	**253**

Dilution factor= 15

Viable cell density = 36.6×10^6 per mL

Total cell density = 37.95×10^6 per mL

Viability = 96.44%

The total volume of PBMCs suspension was 1.3mL, therefore total number of viable cells from 40mL blood was 47.58 million

Morphology of cultured MoDCs

Starting from day 2 to day 6 the morphology of cultured cells was observed and these showed alternation in morphology towards MoDCs. The phenotype of MoDCs was characterized by acquiring and then analysing cells through flow cytometer. There was significant change in morphology of these cells in culture flask, there appear

small projections of plasma membrane and with time these becomes larger (the dendrites), which is the morphological significance of DCs

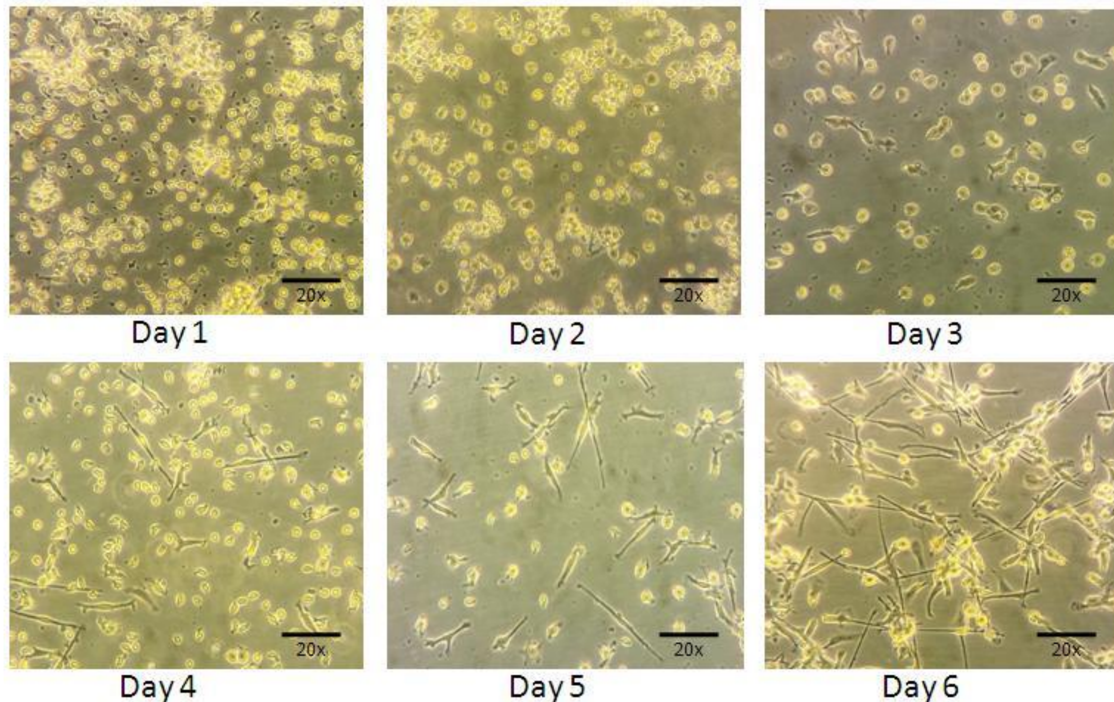


Fig 1; Microscopic evaluation of morphology of MoDCs from Day 1- 6

CFSE signal sustainability

To check the signal sustainability, PBMCs isolated from human blood were labelled with CFSE dye in a six well plate and the cells from each well were collected at different intervals as mentioned above and fixed onto glass slide by 4% paraformaldehyde. These smears were observed under fluorescent microscope showing fluorescence from 6 hr until 96 hours. This suggests the CFSE signal retain for long time inside cells. The same experiment was also performed with MoDCs for 24 & 48 hr.

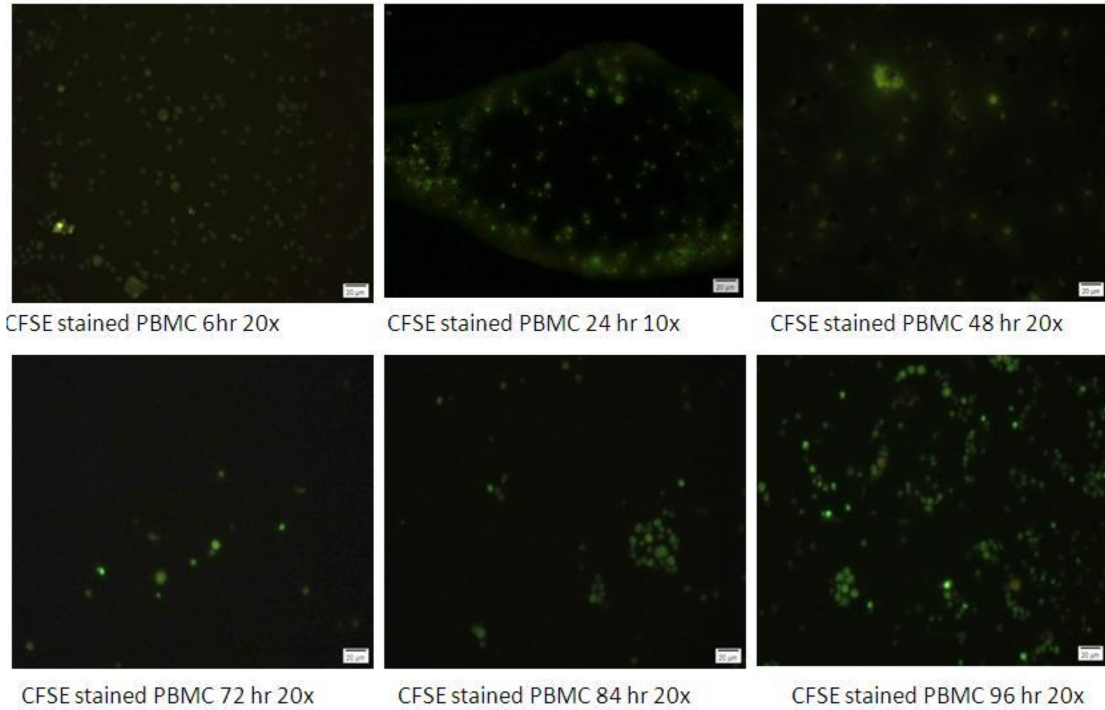


Fig 2; SustainabilityofCFSE (10µM) signal in PBMCs

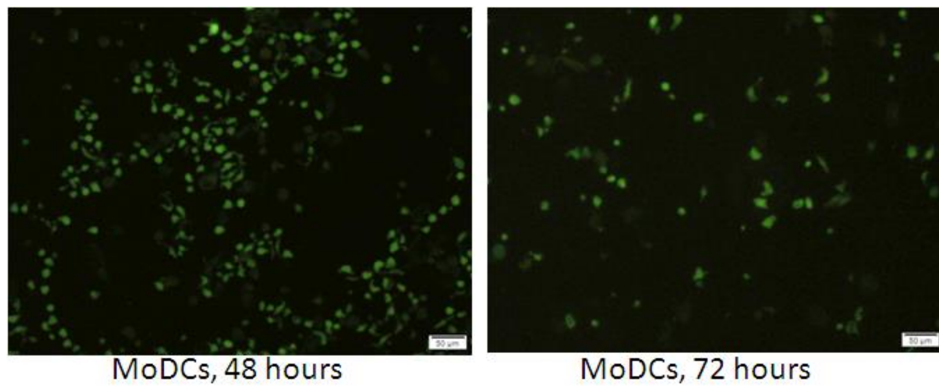
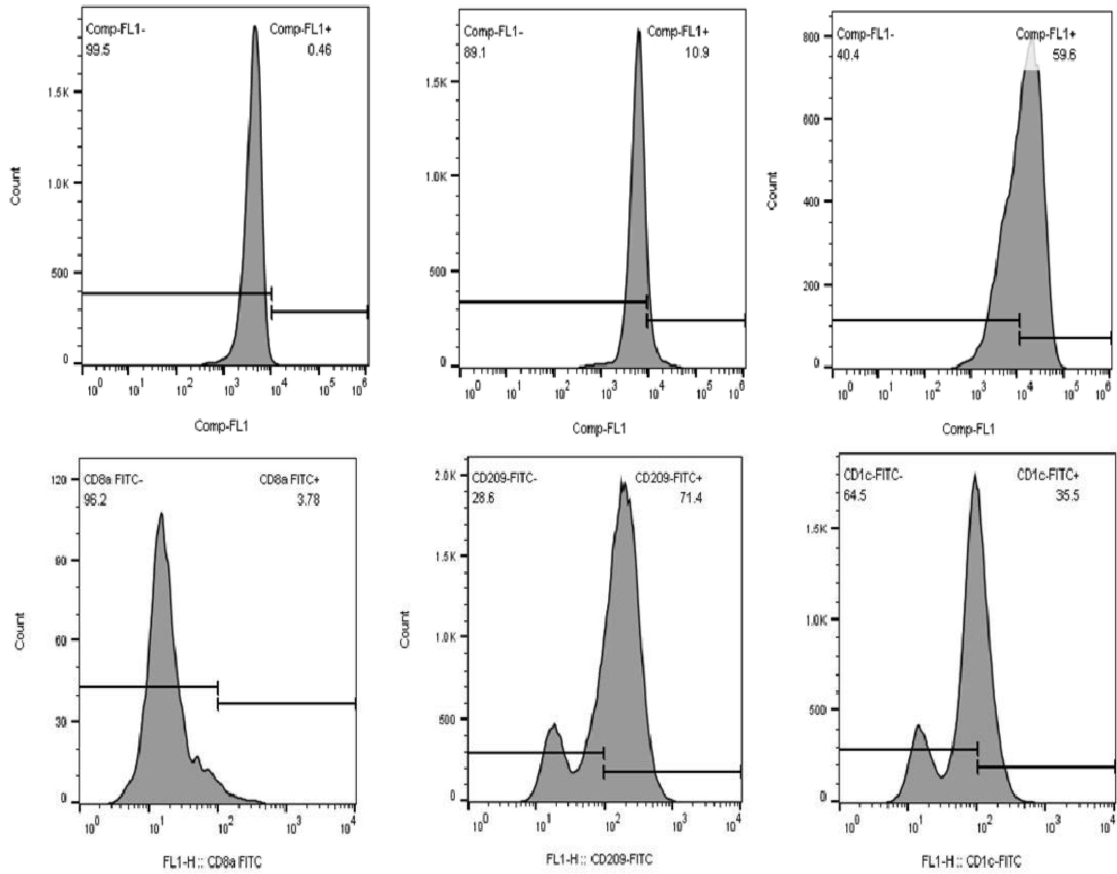


Fig 3; Signal sustainability of CFSE (25µM concentration) in MoDCs

Phenotypic characterization of MoDCs

CD14+ monocytes were cultured for 5-6 days and then characterized with respect to their phenotype flow cytometry after staining with anti-human FITC-CD209 and anti-human

FITC-CD1c monoclonal antibodies. The results are suggestive of the differentiation of monocytes into MoDCs phenotype confirmed by the expression of CD1c and CD209.



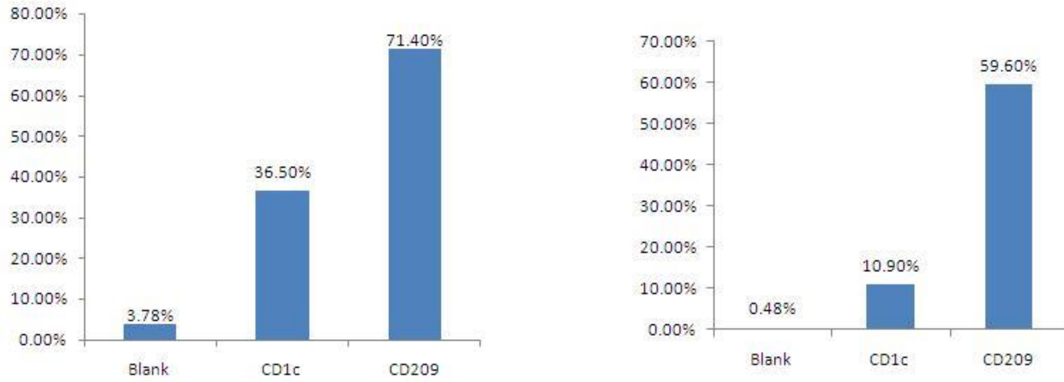


Fig 4; The expression profiling of CD1c and CD209 biomarkers on the surface of monocyte-derived DCs.

Assessment of zeta potential, particle size and polydispersity index of liposome

The microscopic observation showed spherical shape of cld-lip. One identifiable peak represents the homogenous population of liposome of diameter of 573.3nm, and a PDI of 0.395 shows a variation of97% among the population. Zeta potential of -25.8 confirmed the absence of aggregation among the prepared liposomes.

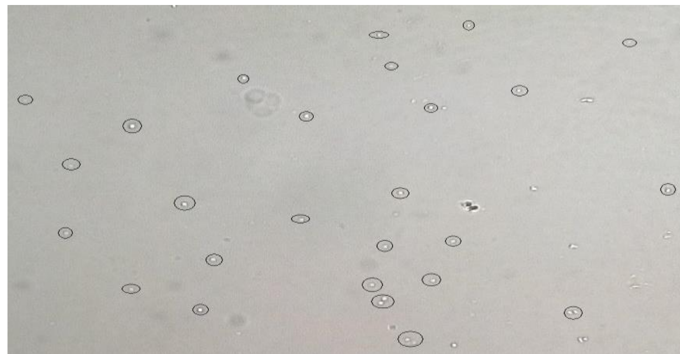
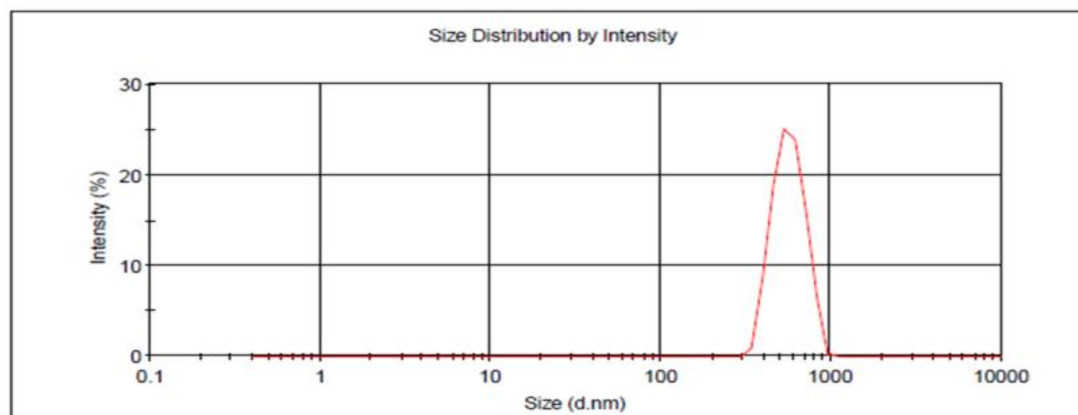


Fig 5; Microscopic visualization of prepared liposomes(40x)

Results

			Size (d.nm):
Z-Average (d.nm):	548.8	Peak 1:	573.3
PdI:	0.029	Peak 2:	0.000
		Peak 3:	0.000

Result quality : Good



Results

			Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV):	-25.8	Peak 1:	0.00	0.0	0.00
Zeta Deviation (mV):	0.00	Peak 2:	0.00	0.0	0.00
Conductivity (mS/cm):	21.4	Peak 3:	0.00	0.0	0.00

Result quality : Good

Assessment of sequestration/partial evasion of labelled MoDCsin different deep-seated tissues of mice

Different organs were extracted from the euthanized mice, thick smears were drawn to observe under fluorescence microscope to determine the sequestration of MoDCs in deep seated tissues. When DC 2.4 (murine cells) were labelled with CFSE and injected to mice. As cells were not found in mouse's periphery, we decided to euthanize animals to see whether cells are sequestered in deep-seated organs such as lymph nodes and lungs after 24 hours. Post 48 and 72 hr there were hardly any cell seen in any of the organs extracted. When MoDCs were labelled with 25µM of CFSE and injected to wild type mice, no CFSE labelled cells were observed from different organs after 24hour. When the animals controlled for their immune-deficient mice

was injected with CFSE labelled MoDCs, these CFSE labelled DCs were found in kidney.

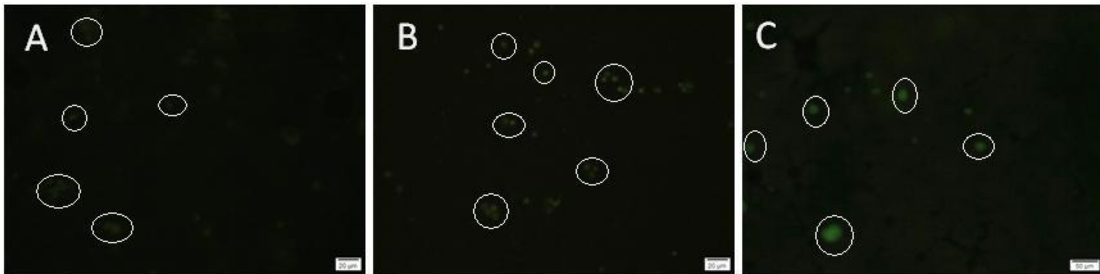


Fig 6 A) CFSE labelled DC2.4 in lung of mice after 24 hr, B)CFSE labelled DC2.4 in lymph node of mice after 24 hr C) CFSE labelled MoDCs in kidney of mice treated with clo-lippost24 hr treatment

DISCUSSION

Present work is an attempt to generate dendritic cell phenotype from human peripheral blood CD14^{+} monocytes by the stimulation with human recombinant GM-CSF (100ng/mL) and IL-4 (25ng/mL).The different concentrations of growth factors have been used by the scientists to generate sufficient number of DCs from CD14 monocytes. PBMCs constitution is as provided; monocytes (10-30%), lymphocytes (70-90%), dendritic cells (1-2%), NK cells (5-20%).The monocytes were isolated from the PBMCs by the conventional adherence techniques. Of all the cells of from PBMCs, monocytes are reported to be activated (during pipetting, shaking and centrifugation) and have the abilities to adhere to the plastic culture flask due to the expression of β2-integrin family. Affinity chromatography experiments revealed that the Mac-1 and p150,95 integrins could be isolated from monocyte-differentiated HL-60 cells on a denatured protein-Sepharose column(*Davis GE, 1992 Jun;200(2):242-52*). The differentiation of monocytes into DCs is dependent upon GM-CSF and IL-4.GM-CSF plays a crucial role in the proliferation, survival and differentiation of dendritic cells through various pathways (JAK/STAT, NFkB, MAPK and PI3K) whereas IL-4 prevents the pathway of macrophage differentiation from monocytes.(*Lianne van de Laar et al,2016)*

Our results show the alteration in morphology of monocytes towards dendritic cell like population day 3of cultivation. The generated dendritic cells were characterized by flow cytometry to check the expression of surface markers [CD209(DC-SIGN) and CD1c (BDCA1)] on DCs. The significant expression of said markers is suggestive of the successful generation of dendritic cells from CD14 monocytes*(Christopher W. Cutler et al, Journal of Leukocyte Biology, Volume 94, August 2013).* CD 209 showed greater expression levels than CD1c and advocates it as one of the suitable and inevitable markers required for the characterization of MoDCs. The CD209- and CD1c- cell populations do not express these surface markers and considered as monocytes. Further, during the differentiation they start differentiatingin to DCs. The phenotypic characterization via flow confirmed that cells expressing CD209 and CD1C are indeed monocyte derived DCs. Also, we did not see any negative effect of the anticoagulants used (heparin and EDTA)during the blood collection on the *ex-vivo* generation of DCs.*(Sally Ahmed El-Sahrigyet al, 2015*)

The frequency (on each and every other day) of replenishing medium supplemented with GM-CSF and IL-4 does have an impact on the differentiation of monocytes into MoDCs. The use of antibiotics in culture medium is avoided to refrain the issue of antibiotic resistance (*NicoVanRooijen et al,1989*). We however used antibiotics to rule out the possibility of bacterial contamination. The mechanical stimulus such as vigorous pipetting, shaking etc, might stimulate maturation of MoDCs and one of the reasons to have morphologically distinct MoDCs. Monocyte derived dendritic cells possess greater adherence ability because which is confirmed by the more time taken for incubation (5-8min) with trypsin-EDTA to get de-adhere. The greater adherence, due to higher expression of adhesion molecules, and larger size of is suggestive of the transformation of CD209+ and CD1c+generated MoDCs.

Ex-vivo generated MoDCs were labelled with 25µM CFSE dye which binds covalently to the lysine residues of proteins and retains for a longer periods. *CMFDA (5-chloromethylfluorescein diacetate)* is also reportedly used to track dendritic cells in a humanized mouse model (Manuscript under review, Tyagi *et al, 2016*). CFSE labelled DC2.4 and MoDCs were assessed for the signal sustainability and our results

are indicative of high durability of CFSE inside the cells (for96 hr). The CFSE labelled DC 2.4 showed less intensity of signals emanated from labelled cells because lesser concentration (10μM) of CFSE was used. These CFSE labelled cells (3 millions) were injected into wild type mice which have been controlled, by clo-lip treatment, for the non-adaptive immune responses especially the cells of monocyte macrophage lineage. The animals were euthanized and deep-seated organs were extracted after 24hr, 48hr and 72hrto see whether cells are sequestered because we hardly see any huMoDCs in mouse periphery after5-6 hrs post-injection. The murine DCs (DC2.4) were injected into mice and showed compatibility with mice cells. The administered cells were seen in lymphnodes and lungs after 24 hr post treatment. Further, after 48hr and 72 hr, we did not see any cells neither in circulation nor in organs. When CFSE labelled *ex-vivo* generated MoDCs were injected into wild type mice, no cells were tracked in any of the deep seated organs. It may be reasoned due to highly recruited monocyte and other phagocytic cells, which are reported to clear cells from mouse(*Tyagi et al,2010)*

To suppress these highly recruited monocytes and other phagocytic cells, we injected 200μL of clodronate (disodium clodronatetetrahydrate; TCI Chemical Pvt. Ltd.) loaded liposome throughintraperitoneal route of administration. Clodronate is impermeable through plasma membrane and it has a very less half-life in mouse circulation. Monocytes and macrophages actively take up the clo-lip, the clodronateis released inside the cytoplasm of these cells, when a threshold limit of clodronateis reached and induces phagocytosis of these cells which, in turn, leads to selective apoptosis of macrophages/monocytes (*Nico Van Rooijenet al).* We injected clo-lip2-3 at an interval of 1-2 days. On 7th day of cell culture, CFSE labelled MoDCs were harvested, phenotyped and administered to animals. After24 hrs, mice were euthanized to track the traversal of MoDCs and possible sequestration which mice cells may have employed to escape active immuneresponse of the host. The immunofluorescence assays performed on organs samples partially indicate the evasion/escape mechanism employed by MoDCs to last-longer in kidney and other organs. Also, mice injected with clo-lip were seen to present the enlarged spleen,

lymphnodes and liver. The larger size of mentioned organs is due to clo-lip mediated inflammation. Our results are in agreement to earlier reports (*Tyagi et al,2010)*

Conclusion &future perspectives

Present study validates the differentiation of CD14 monocytes into CD1C and CD209+ dendritic Cells in presence of stimulus in GM-CSF and IL4 *in vitro*. Also, generated MoDCs showed characteristic phenotype of dendritic cells which was then confirmed by surface phenotyping through flow cytometry. The partial sequestration is one of the striking findings of our work since the administered MNs &MoDCs were not seen in mouse's periphery but cells were traced in different organs viz. Lungs, kidney and lymph node.

MoDCs can be implied in personalized vaccine for developing therapeutic interventions against cancer, and as adjuvant for the development of sub-unit vaccine. MoDCs are the only cells that efficiently presenting activated virus, and therefore the efficacy of the new generation of attenuated vaccines could be improved by specific targeting to DCs[*Dirk R. Van Bockstaele et al,2001*].

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