Studies on differentiation and functional maturation of dendritic cells in cancer microenvironment

By

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Vipul Kumar Pandey

DECLARATION

I, hereby declare that the investigation presented in the thesis has been carried out by me. The work is original and has not been submitted earlier as a whole or in part for a degree / diploma at this or any other Institution / University.

Vipul Kumar Pandey

List of Publications arising from the thesis

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SYNOPSIS

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SYNOPSIS

Introduction

The cancer microenvironment is a unique niche surrounding the tumor which constitute both cellular (immune cells fibroblasts, adipocytes and endothelial cells) and non-cellular (extracellular matrix, soluble cytokine, growth factors and tumor vasculature) components (*1*). Although cancer microenvironment is often infiltrated by large number of immune cells, they are not only dysfunctional and fail to generate an effective anti-tumor response but often manipulated by cancer to support its growth (*2*). Dendritic cells (DC), a key component of immune system's antitumor response facilitate both the innate and adaptive response against cancer cells (*3, 4*). Suppression of dendritic cell function in cancer plays a major role in inhibition of

immune responses and disease progression and also limits the success of cancer immunotherapy (*5*). Many studies on DC and cancer microenvironment focus on DC present in vicinity of cancer. But how cancer microenvironment and its associated factors affect the differentiation of DC from progenitor is an open question. This question becomes more pertinent in immunotherapy where patients own immune system is used to fight cancer. DC progenitors reside in bone marrow and they attain a significant lineage specific commitment inside bone marrow only. Recently, a consortium of Immunologists and computational biologists under "The Immunological Genome (ImmGen) Project" have deciphered the transcriptional network of DC development from early progenitors to different types of DC (*6*).

The hypothesis of this study is that cancer and its associated factors affect the DC development at the progenitor level. The specific objectives of the study are:

1) To study the role of cancer microenvironment on differentiation and maturation of DC.

2) To assess the differential miRNA expression in DC affected by cancer microenvironment.

3) To study the effect of progenitor cell irradiation on subsequent differentiation and maturation of DC.

The work embodied in this thesis is dived into four chapters: Chapter 1: General introduction and review of literature. Chapter 2: Materials and methods. Chapter 3: Results. Chapter 4: General discussion and conclusion. The results chapter is further subdivided into 3 sections. 3.1: Effect of cancer microenvironment on differentiation and maturation of DC. 3.2: Differential expression of miRNA in cancer induced DC

dysfunction. 3.3: Effect of progenitor cell irradiation on differentiation and maturation of DC.

Chapter 1. This chapter gives an overview of the hallmarks of cancer and the contribution of cancer microenvironment, particularly the immune system in promoting cancer. This will be followed by dendritic cells and its dysfunction in cancer (*1,* 7-9). There are different subtypes of DC, both myeloid and lymphoid depending upon their origin, location and function. (*10*). Myeloid DC are called classical DC (cDC) while lymphoid DC are called plasmacytoid DC (pDC). Classical DC are either lymphoid tissue resident ($CD8^+$,$CD8^-$) or non-lymphoid tissue resident DC ($CD103^+$ and $CD11b^+$). The role of CD8 + DC, which are most crucial in anti-tumor immune response because of their cross-presentation ability, will be highlighted. The several lineages of DC are determined by presence and absence of specific transcription factors. Among many others, major players are Batf3, Zbtb46, E2-2, Id2, PU.1, IRF7 and IRF8 (*11*). Zbtb46 (zinc finger transcription factor 46) expression is associated with the commitment of common dendritic cell precursor to the cDC lineage and thus Zbtb46 can serve as a useful marker for distinguishing cDCs from other tissue phagocytes.

DC dysfunction is most often associated with progression of cancer. Being heterogeneous, they are highly susceptible to different factors secreted or associated with cancer. (*12*). Many studies have reported that miRNA play an important role in differentiation of DC (*13*). MicroRNAs (miRNAs) are small RNA molecules that regulate gene expression and thereby influence cell fate and function. The generation of miRNAs proceeds via a specific pathway involving the RNase Dicer that produces

RNA duplexes of ~21 bp in length. miRNA regulate the gene expression by binding to target mRNA and either degrade it or repress the translation (*14*). miR-132 and miR-147 were highly expressed in immature and mature DC but not found in progenitor cells (*15*). Studies have also reported the role of miRNA in lineage commitment. miR-22 was highly expressed in cDC (both $CD8^+$ and $CD8^-$) compared with pDC (*16*). Similarly miR-126 positively regulated differentiation of progenitors to pDC (*16*) and also inhibition of miR-221 expression in BMDC progenitors led to the differentiation of pDC rather than cDC (*17*). In this context, it is very crucial to explore the role of miRNAs in cancer microenvironment induced DC dysfunction.

In addition to the effects of cancer microenvironment on dendritic cell function, the various treatment modalities used to treat the cancer, including ionizing radiation may affect dendritic cell function. In some recent studies, it has been shown that radiation therapy instead of being immunosupressive may enhance expression of cancer-associated antigens, diminish regulatory T-cell activity and activate dendritic cells through Toll-like receptor (TLR)-dependent mechanisms (*18*). In this context, the mechanistic understanding of the effect of radiation on differentiation as well as maturation processes of DC will be helpful to improve anti-tumor immunity and clinical response to therapy.

Chapter 2. This chapter describes in detail the different materials and the methodology of the experiments used in this study. Detailed protocols of the procedures have been categorized in to 4 subsections. (i) Cell culture techniques that include cell line maintenance, sub-culturing protocol, bone marrow cells isolation, dendritic cells differentiation, preparation of tumor conditioned media, cell

proliferation assay, cross presentation assay, antigen processing and phagocytosis assay, siRNA mediated knock down procedure. (ii) Quantitative and semi-quantitative techniques which include ELISA, electrophoresis, western blotting, flow cytometry. (iii) *in vivo* techniques which include generation of mice lymphoma model, treatment of mice, tumor measurement. (iv) miRNA isolation, sequencing and analysis.

Chapter 3. This chapter includes the work carried out in this study. It is divided into 3 sections.

3.1: Effect of cancer microenvironment on differentiation and maturation of DC:

(a) Tumor conditioned media suppressed differentiation of DC: In this chapter, the results describing the effect of tumor conditioned media (TCM) from EL4 lymphoma cells on bone marrow derived dendritic cells (BMDC) as well the splenic dendritic cells from tumor bearing mice will be presented. Phenotypic maturation status of DC studied using expression of maturation markers CD40/80/86 and IA/IE revealed down regulation under *in vitro* and *in vivo* conditions. Functional competency of DC was assessed by phagocytosis, antigen processing as well as allogenic T cell proliferation, which were suppressed in DC (TCM).There was decreased cross-presentation ability of DC (TCM) as well which was evaluated by two methods. (1) Presentation of SIINFEKL peptide (Ova 257-264) in context of MHC I (H2-b). (2) Activation of CD8+ B3Z hybridoma cells, upon binding to SIINFEKL/MHC I (H2-b) complex.

(B) TCM induced immunosuppressive IL-10 by DC: TCM did not contain detectable amount of IL-10. However, it induced secretion of immunosuppressive IL-10 and inhibition of IL-12 from day 6 of DC differentiation. TCM induced upregulation of IL-10 was mediated through MEK/ERK/CREB signaling pathway. Treatment with ERK inhibitor PD98059 treatment down regulated ERK and CREB phosphorylation in DC (TCM) as well as down regulated IL-10, but did not restore TCM induced suppression of maturation markers indicating that it may not be the primary factor responsible for DC dysfunction. Further, addition of exogenous IL-10 also did not mimic the effect of TCM during differentiation indicating that TCM modulated other crucial proteins responsible for DC impairment.

(c) Role of lineage specific transcription factors: The expression of several lineage specific transcription factors which play an important role at different stages of DC development were evaluated in DC (TCM). Expression level of transcription factors Id2, Zbtb46, Bcl6, E2-2 and Batf3 were analysed by real time PCR in DC, DC (TCM) as well as bone marrow cells (BMC) of control mice and TBM. Among all the transcription factors, the level of Zbtb46 and Bcl6 were consistently downregulated in DC (TCM) and BMC of TBM as compared to their respective controls. This was further confirmed by western blot analysis and flow cytometry. The expression of maturation markers CD 40/80 and MHC II were significantly down regulated when Zbtb46 was knocked down by siRNA treatment during DC differentiation. Downregulation of Bcl-6 also followed a similar pattern.

(D) Role of prostaglandin in cancer induced suppression of DC differentiation

(i) Effect of NS-398 on TCM induced DC dysfunction (*in vitro)*: Since PGE2 has a prominent role in induction of IL-10 by DC (*19*), it's role was explored in DC (TCM) dysfunction. EL4 cells were treated with NS-398 a selective COX-2 inhibitor and this TCM(NS-398), when used for differentiation of DC did not show similar inhibitory effects as TCM. This was also confirmed by downregulation of DC immunogenicity by synthetic PGE2 similar to TCM treatment. To rule out the possible role of DC derived prostaglandin in DC dysfunction, NS-398 was added along with TCM in DC (TCM). This did not abrogate the TCM induced downregulation of maturation markers on DC.

(ii) Effect of NS-398 on TCM induced DC dysfunction (*in vivo*): To evaluate the effect of NS-398 *in vivo*, mice were injected with EL4 cells s.c. and divided into 3 groups: TBM (control), TBM (vehicle) and TBM (NS-398) along with no tumor (NT) control. TBM (NS-398) mice were treated with NS-398 from day 1 onwards to day 15. TBM (control) had elevated levels of PGE2 level as compared to NT (control) which was mitigated in TBM (NS-398). In addition, phenoptypic and functional status of DC in TBM (NS-398) was comparable to no tumor (NT) control. These observations revealed restoration of DC maturation following NS-398 treatment and was accompanied by significant reduction in tumor burden in TBM (NS-398) as compared to TBM (control) ($p=0.008$) and TBM (vehicle) ($p=0.01$).

(iii) Effect of NS-398 in cancer bearing immunodeficient mice: Experiments in immuno-competent TBM had suggested that anti-tumor effect of NS-398 was through dendritic cells. To further confirm this hypothesis, NS-398 was tested in EL4 lymphoma grown in immuno-incompetent SCID (severe combined immunodeficiency)

mice. No difference was observed in phenotypic status of BMDC and there was variable response in splenic DC between the different treatment groups. There was no significant difference in tumor size between TBM, TBM (vehicle) and TBM (NS-398).

(iv) Effect of NS-398 along with chemotherapeutic drug camptothecin in TBM: Treatment with NS-398 did not have direct cytotoxic effect on EL4 cells and probably reduced tumor burden mainly by restoring immune function especially DC. In order to identify potential synergistic effect of NS-398, it was used along with cytotoxic drug camptothecin (CPT). Maturation markers analysis showed similar restoration of DC function in TBM (CPT+NS-398) and TBM (CPT) compared to TBM and TBM (veh). Tumor volume was significantly reduced in TBM (NS-398) ($p<0.05$) and TBM (CPT) ($p<0.007$) when compared to TBM control. Tumor volume was further reduced significantly in TBM (NS-398+CPT) when compared to TBM (NS-398) ($p=0.004$) as well as when compared to TBM (CPT) ($p=0.011$).

3.2: Differential expression of miRNA in cancer induced DC dysfunction: Differential expression of miRNA was studied in differentiated DC (immature as well as mature) as well as in progenitors (BMC and BMC (TBM)). miRNA was isolated from iDC, iDC (TCM), mDC, mDC (TCM), BMC (NT) and BMC (TBM), libraries were prepared and global miRNA sequencing was done using Illumina HiSeq 2500 platform of SBS version 4. Sequencing data were analyzed using miRDeep2 module, which processes the raw sequencing output from the illumina platform and maps the processed reads to the reference genome and provide known and novel miRNAs. A comparative analysis was carried out between iDC and iDC (TCM), mDC and mDC

(TCM), BMC and BMC (TBM). Five known miRNA were upregulated and 4 were downregulated significantly in iDC (TCM). Similarly, between BMC and BMC (TBM), 12 known miRNA were upregulated while 23 known miRNA were downregulated significantly. Two differentially expressed potential novel miRNA were also identified in BMC (TBM) when compared with BMC (NT). From RNA sequencing data, 6 relevant miRNA were chosen and their expression profile was verified using quantitative PCR both in iDC TCM vs iDC and mDC vs iDC. In RT-PCR analysis miR-155-5p, miR-155-3p, miR-146a-5p, miR-365-2-5p and miR187-3p showed similar pattern of differential regulation as RNA sequencing analysis.

3.3: Effect of progenitor cell irradiation on differentiation and maturation of DC: Dendritic cells derived from tumor bearing mice showed compromised phenotypic and functional maturation. Experiments were therefore carried out to find out if treatment of progenitor cells with any agent could mitigate these effects. BMDC differentiated from irradiated bone marrow precursor cells (0.5 Gy and 1.0 Gy) showed increased expression of CD 40, CD 80, CD 86 and MHC II receptors when compared to BMDC from un-irradiated precursor. DC from irradiated precursors also secreted higher amount of IL-12. DC from irradiated precursor (0.5 Gy and 1.0 Gy) showed higher expression of lineage specific transcription factors Id2 and Zbtb46 as compared to control. As the DC from irradiated precursors showed increase in immunogenicity and those treated with TCM demonstrated phenotypic and functional suppression of DC, it was pertinent to explore whether irradiated precursor cells could overcome cancer microenvironment induced DC dysfunction. Irradiated precursor cells (1 Gy) were cultured in presence of TCM and were differentiated in

BMDC following the standard protocol. Flow cytometry analysis showed that when compared to DC (control), there was an increase in expression of maturation markers in DC (1 Gy) and decrease in DC (TCM) and this decrease in DC (TCM) was mitigated in DC (1 Gy+ TCM). Although the expression level of maturation markers in DC (1 Gy+ TCM) was higher, it was still lower than DC (1 Gy).

This increase in immunogenicity of DC derived from irradiated progenitors was probably due to the low levels of apoptotic cells generated due to ionizing radiation exposure. This was confirmed with experiments performed in the presence or absence of apoptotic cells, removal of apoptotic cells followed by differentiation into DC as well as by *in vivo* experiments.

Chapter 4. This chapter covers the discussion of the results and conclusion of our studies. Cancer microenvironment renders dendritic cells phenotypically and functionally dysfunctional. The key finding of the study is that the cancer not only affects the DC in vicinity but also the DC progenitor cells present at distant site by downregulating the lineage specific transcription factor Zbtb46 through prostanoids secreted by cancer cells (EL4 lymphoma). Many studies have reported the role of COX-2 in cancer ranging from progression to immunosuppression (*20*-*22*). In our study, we have shown that inhibition of COX-2 in tumor cells can mitigate the effect of cancer microenvironment on DC differentiation *in vitro* and treatment of tumor bearing mice with COX-2 inhibitor can restore DC function and concomitant reduction in tumor burden also. This finding was further validated by no decrease in tumor burden in EL4 lymphoma bearing SCID mice. This study has also identified a novel target, the transcription factor Zbtb46 for prostaglandin mediated dysfunction of DC.

Down regulation of Zbtb46 by the cancer derived prostaglandins or by siRNA during differentiation was primarily responsible for dampening of the DC function. Though IL-10 was also produced, it was effective only during maturation. Recent advancements in cancer biology have helped to design new immunotherapy protocols. However success of standalone immunotherapy of cancer is limited due to restriction of immune system to effectively eliminate rapidly growing cancer cells (*23*). Thus, it is important to formulate combinatorial therapy protocols where cancer is treated by direct killing of cancer cells as well as by restoration of immune system to check any recurrence. In this study, it was shown that combination treatment with NS-398 along with cytotoxic drug CPT not only restored immune system similar to NS-398 treatment but also cancer reduction was much more significant in comparison to both CPT and NS-398 treatment alone. This opens up a new possibility of immunotherapy protocol where use of immunotherapeutic drugs actually helps in decreasing chemotherapy doses and thus reduces the chances of various side effects of chemotherapy.

We have observed that role of the lineage specific transcription factors plays a crucial role in DC (TCM) and BMC (TBM). Many studies have shown the importance of miRNA in regulating the transcriptional network of an immune cell especially under stress conditions (*14*). In this study, the global miRNA sequencing has shown differential expression of many known and novel miRNA in DC (TCM) and BMC (TBM) compared to their respective controls. Bioinformatic analysis has shown that miR-486a-5p which was downregulated in BMC (TBM), play an important role in mice erythroid precursors (*24*). Similarly, miR-146a-5p which was upregulated in DC

(TCM) compared to DC has been reported to negatively regulate DC activation and maturation (*25*). Likewise, miR-132-3p, which was differentially regulated in DC (TCM) compared to DC is involved in regulation of sonic-hedgehog signaling which is in turn is regulated through prostaglandins (*26*). These observations are in tandem with our finding about role of prostaglandin in DC dysfunction. Comparison of miRNA expression in iDC and mDC showed upregulation of miR-132-5P and downregulation of miR-504-5p and miR-378d. Interestingly, these miRNAs also followed same expression pattern in mDC (TCM) vs mDC. In this context, this pattern of similar miRNA profile between iDC and mDC (TCM) suggest the probable role of these miRNA in TCM induced downregulation of maturation markers in DC.

In most of the DC based immunotherapy protocols, $CD14^+$ monocytes are isolated from patient blood and differentiated into dendritic cells (*6*). These monocytes are already affected by cancer microenvironment. It raised a possibility that DC generated from such monocytes may not be fully competent to perform the function. This study has shown that TCM induced DC dysfunction was overcome when progenitors were irradiated. This suggests a possible convergence of mechanism which regulates DC dysfunction as well increase in DC immunogenicity and can be potentially used to improve DC based cancer immunotherapy outcome.

Publication in Refereed journal:

1. Published: 'COX-2 inhibitor prevents cancer induced down regulation of classical DC lineage specific transcription factor Zbtb46 resulting in immunocompetent DC and decreased tumor burden' Vipul K. Pandey, Prayag J. Amin, Bhavani S. Shankar, Immunology Letters, 2017, 184:(23 -33).

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AP-1	Activator Protein-1
APCs	Antigen presenting cells
batf3	Basic Leucine Zipper ATF-Like Transcription Factor 3
Bcl6	B-cell Chronic Lymphocytic Lymphoma 6
BMDC	Bone Marrow Derived Dendritic Cells
bZip	Basic Leucine Zipper 1
CD	Cluster of Differentiation
CFSE	Carboxy Fluorescein Diacetate Succinimidyl Ester
COX	Cyclooxygenase
CPT	Camptothecin
CREB	cAMP Response Element-Binding Protein
CSF	Colony Stimulating Factor
CTLA4	Cytotoxic T-Lymphocyte Antigen 4
DC	Dendritic cells
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-Linked Immunosorbent Assay
ERK	Extracellular Signal-Regulated Kinase
FADD	Fas-Associated Protein with Death Domain
FBS	Fetal Bovine Serum
GM-CSF	Granulocyte Monocyte Colony Stimulating Factor
HMGB1	High Mobility Group Box 1
Id2	Inhibitor of DNA binding 2

IFN	Interferon
IFNγ	Interferon Gamma
IKK	IκB kinase
IL	Interleukin
IL-10	Interleukin-10
IL-12	Interleukin -12
IL-1β	Interleukin-1β
IL-4	Interleukin-4
IL-6	Interleukin-6
iNOS	Inducible Nitric Oxide Synthase
IR	Ionizing Radiation
LPS	Lipopolysaccharide
MAPK	Mitogen-Activated Protein Kinases
MHC	Major Histocompatibility Complex
MMP	Matrix Metalloproteinases
NF-κB	Nuclear Factor Kappa B
NKC	Natural Killer Cells
NSAIDs	Non-steroidal Anti Inflammatory Drugs
PAMP	Pathogen-Associated Molecular Patterns
PBS	Phosphate Buffered Saline
PD-L1	Programmed Death-Ligand 1
PGE2	Prostaglandin E2
PI3K	Phosphatidylinositol 3-Kinase

RISC	RNA-Induced Silencing Complex
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute
sDC	Splenic dendritic cells
siRNA	Small Interfering RNA
SOCS1	Suppressor of Cytokine Signaling 1
SOX4	SRY-related HMG-box
STAT	Signal Transducers And Activators Of Transcription
TBM	Tumor Bearing Mice
TC	Cytotoxic T Cells
TCF4	Transcription Factor 4
TCM	Tumor Conditioned Media
TCR	T Cell Receptor
TGF-β	Transforming Growth Factor Beta
TH	Helper T cells
TILs	Tumor-Infiltrating Lymphocytes
TLR	Toll Like Receptor
TNFα	Tumor Necrosis Factor Alpha
Treg cells	T-Regulatory Cells
VEGF	Vascular Endothelial Growth Factor
Wnt	Wingless-related Integration Site
Zbtb46	Zinc Finger And BTB Domain Containing 46

CHAPTER 1

INTRODUCTION

1.1 Introduction of cancer:

Cancer is a group of diseases characterized by uncontrolled autonomous cell growth after the collapse of boundaries that regulate cellular differentiation, proliferation, function and death (1). Cancer is a highly heterogeneous disease and though the underlying reason behind all types of cancers is the occurrence of numerous genetic alterations (*2*), they also harbor global epigenetic abnormalities (*3*). The development of cancer, referred to as carcinogenesis is a dynamic process that depends on various factors and has a spatial and temporal evolution resulting in enormous diversity. The process of carcinogenesis, at the cellular level, is predominantly an irreversible and multistage process. To understand the mechanism of carcinogenesis, German zoologist Theodor Boveri in 1914 had postulated the theory of somatic mutation in his book 'On the Origin of Malignant Tumors' that states: (i) cancer is a problem of cell proliferation and (ii) cancers are due to abnormal chromosomal rearrangements (*4*). However somatic mutation theory (SMT) did not get due attention till late 20th century when structure of DNA was deciphered along with process of replication. On the other side, there are other theories like tissue organization field theory which reject the SMT, the premises of which are that carcinogenesis represents a problem of tissue organization not solely somatic mutation (*5*). However, most of the cancer research till today is based on the very idea of somatic mutation and the process of carcinogenesis. The transformation process of carcinogenesis in which a normal cell is transformed into a malignant cell consists of three stages: initiation, promotion, and progression (*6*).

1.1.1 Different stages of cancer:

(a) Initiation: During the initiation phase of carcinogenesis, a specific and stable change in a cell's genome either gives the cell a growth advantage over other cells or removes the regulatory barrier over cell proliferation. These mutations either affect proto-oncogenes (gain of function) which codes for various growth factors or their receptors, enzymes involved in signal transduction, and several transcription factors that promote cell growth or tumor suppressor genes (loss of function) which normally suppress carcinogenesis and loss of which would facilitates tumor development. The initiation event can either be triggered by intrinsic factors or extrinsic factors (*7*).

(i) Intrinsic factors: There are many inherent factors like genetic constitution, ancestral history, aging, hormones, chronic inflammation, etc., which may cause genetic instability and ultimately result in initiation of cancer. Many incidences of cancers related to breast, endometrium, ovary, prostate, testis, thyroid and osteosarcoma, share a unique mechanism of carcinogenesis which is based on neoplasia of hormone-responsive tissues (*8*). National Institute of Environmental Health Sciences (NIEHS) has recently added estrogen to the list of known cancer-causing agents as epidemiological studies have conclusively shown a positive correlation between estrogen and breast cancer (*9*). Similarly, studies have also shown that estrogens as opposed to progestins can increase the risk of endometrial cancer (*10*). Cellular senescence and deficit in immune responses are among many probable reasons responsible for age associated increase in cancer risk. Numerous studies have demonstrated positive correlation between process of aging and cancer incidences. For example, 60% of all types of cancer incidences

and 69% of all cancer deaths occur in just 13% of the U.S.A. population aged 65 years or older (*11*). Inflammation is a normal physiological response of immune system that causes injured tissue to heal. However, many cancers are caused due to chronic inflammation also called “smouldering inflammation” or “wounds that do not heal” by Rudolf Virchow in 1858 (*12*). Chronic inflammation can be caused secondary to infection or by many conditions like chronic inflammatory bowel diseases, such as ulcerative colitis, rheumatoid arthritis, autoimmunity, obesity which may ultimately result in DNA damage and initiate cancer.

(ii) Extrinsic factors: Malignant transformation of a normal cell may be initiated by external factors such as diet and lifestyle, smoking, use of alcohol, several infections and contact with various carcinogenic agents. These include factors which directly initiate mutation or those which indirectly affect genomic instability and result in initiation of malignancy. Carcinogenic agents can be physical agents like radiation (gamma and UV rays), or different types of chemicals like ethidium bromide, formaldehyde, asbestos, benzene, tobacco and heavy metals like arsenic etc (*13*).

Many times, a combination of intrinsic and extrinsic factors can act in synergy resulting in rapid initiation of cancer. For example, potential of UV rays to cause cancer increase many fold if the person is suffering from congenital DNA repair deficiency, like xeroderma pigmentosum. Enhanced susceptibility to cancer is also known in individuals with DNA repair deficiencies such as Bloom’s syndrome and ataxia telangiectasia mutation (*14*). In many cases, foreign molecules which are naturally not carcinogenic are converted to carcinogenic agents by metabolism (*15*).

(2) Promotion: Tumor promotion is the induction of clonal proliferation of initiated cells and can be due to several growth factor receptors, signalling molecules, transcription factors or physical and chemical agents (*6*). It is the stage between premalignant cellular state and cancer and is the process of stepwise transformation of an initiated cell to neoplasm and to malignancy. During the process of progression, daughter cells can acquire additional spontaneous mutations which further resist apoptosis and increase growth rate. This stage is very crucial for early diagnosis and start of cancer therapy.

(3) Progression or Metastasis: Metastasis is the final stage in development of invasive cancer. It involves the spread of cancer cells from the original primary site to other parts of the body and establishment of secondary cancers at those sites. Metastasis starts when tumor cells from primary sites undergo epithelial to mesenchymal transition and move towards regional tissues through blood or lymphatic circulation and can occur in lymph nodes or distant organs (*6*).

1.1.2 Hallmarks of cancer:

During the multistep development, cancer acquires six fundamental properties called the “hallmarks” of cancer: self-sufficient proliferation, insensitivity to anti-proliferative signals, evasion of apoptosis, enabling replicative immortality, inducing angiogenesis, and activation of invasion and metastasis (Fig 1) (*1*). The crucial reason behind all these hallmarks is the genomic instability that generates genetic diversity which drives the acquisition of each of these hallmarks. These hallmarks of cancer are acquired

functional capabilities that allow cancer cells to survive, proliferate, and disseminate. Recent progress in the field of cancer biology have added two more hallmarks (Fig 2): reprogramming of energy metabolism and evading immune destruction (*16*). Cancer cells have been shown to switch to aerobic glycolysis, a phenomenon also called as 'Warburg effect' in which, even in presence of oxygen, cells will use glycolysis pathway for their energy requirements. The other emerging hallmark is 'evasion of immune destruction' which was substantiated by the fact that immunocompromised mice as well as humans showed much higher occurrence of spontaneous cancer (*17*). Many recent studies have shown that there is a strong co-relation between cancer progression and immune dysfunction in almost all kind of cancers (*18-21*). Many mechanisms have been postulated through which cancer cells not only evade immune destruction but also manipulate immune system to promote its own progression. These give rise to a new proposition that tumors are more than just masses of proliferating cancer cells. Instead, they are complex tissues composed of multiple distinct cell types including repertoire of recruited normal cells that participate in interactions with one another. These recruited cells include many types of immune cells and stromal cells and together constitute 'cancer microenvironment' (*22*).

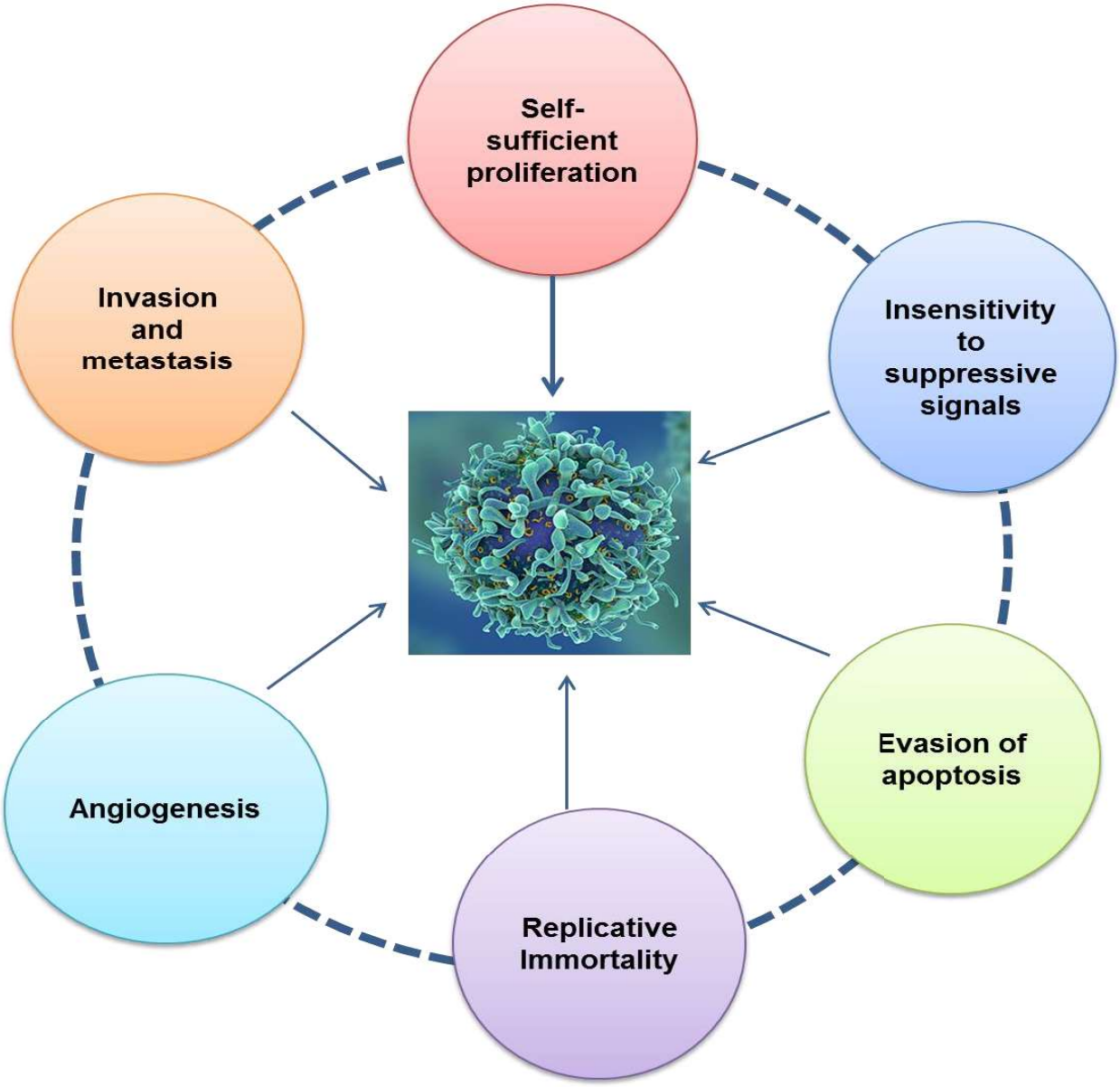


Fig 1: Classic view of 'hallmarks of cancer'. These hallmarks, proposed by Hanahan and Weinberg (*1*), are characteristics of all types of malignancies and gives a framework for understanding the biology of cancer.

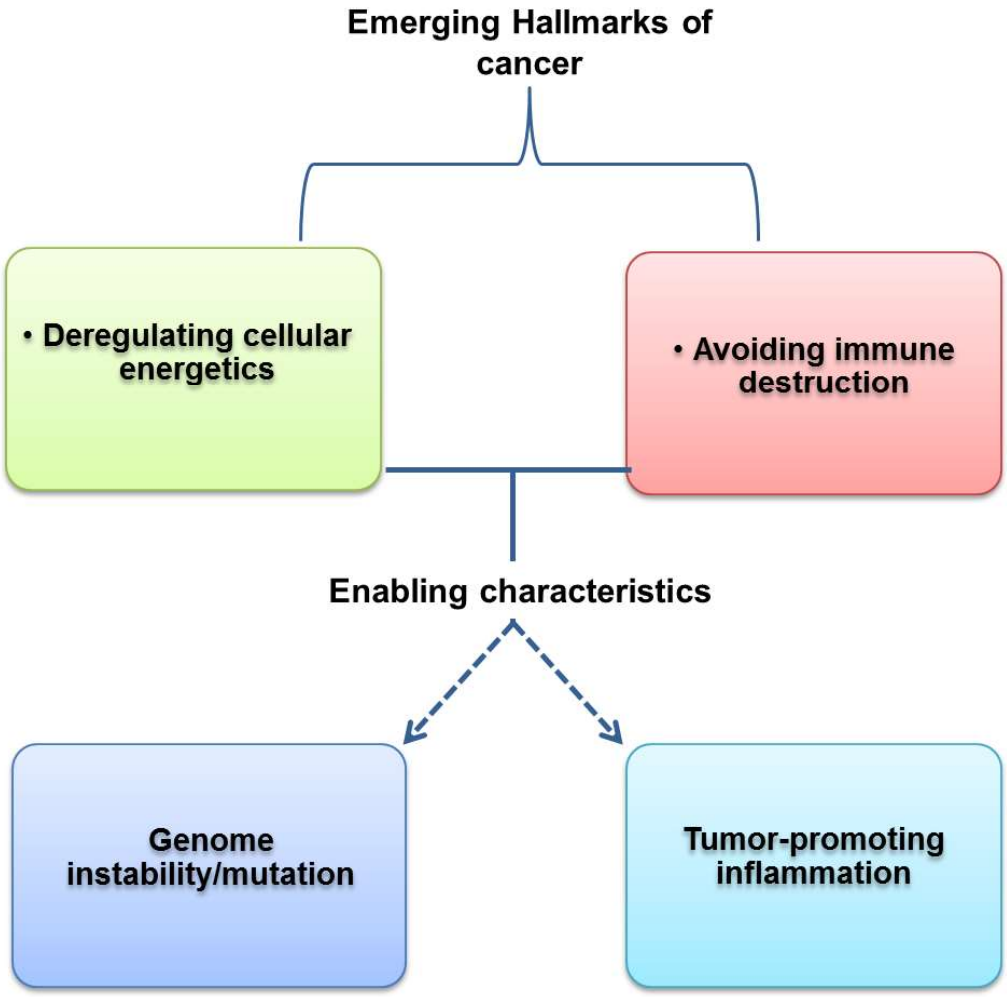


Fig 2: Emerging hallmarks of cancer: Deregulation of cellular energetics and immune escape are two important emerging hallmarks of cancer. Both these hallmarks help in progression of cancer by increasing the genomic instability as well as by supporting the growth of pro-tumorigenic inflammation (*16*).

1.2 Cancer microenvironment:

1.2.1 Introduction of cancer microenvironment: There has been increasing resistance to the traditionally used chemo and radio-therapeutic modalities (*23*). In addition, the understanding of many specific mutations or overexpression of some

proteins like EGFR have resulted in the development of many targeted therapies. Though these drugs were initially successful, there was rapid development of resistance and large inter-individual effects (*24*). Detailed studies to understand the mechanisms underlying the development of resistance revealed the importance of 'niche or microenvironment' surrounding the cancer and its importance in neoplastic cell initiation, malignant progression and metastasis. This holistic understanding of cancer suggests that many well-known mutations in cancer genes affect the cancer milieu and vice versa. In addition to the cancer cells, the microenvironment consists of epithelial cells, fibroblast, endothelial cells, pericytes, immune cells, vascular cells, other stromal cells and various non-cellular components. Broadly these various components can be classified into 4 categories: non-cellular components (extracellular matrix), soluble components (cytokines and growth factors), cells of mesenchymal origin (fibroblasts, myofibroblasts, adipocytes and endothelial cells) and cells of hematopoietic origin (all types of immune cells, both lymphoid and myeloid lineage) (*22, 25*). The interaction between different cell types is quite dynamic in nature and can have both cancer promoting or growth inhibitory effects depending upon various factors (*26*).

(a) Non-cellular components: The extra cellular matrix (ECM) is the most important non-cellular component of tumor microenvironment. It is composed of many types of macromolecules, like collagen, laminin, fibronectin. These proteins are produced by fibroblast and are interlinked in a complex, three-dimensional matrix (*27*). The stroma is separated from epithelium by a specialized kind of ECM called as basement membrane. In a normal tissue, the tissue homeostasis is maintained through cell-cell contact;

however such restraints are overridden by specialized ECM in case of cancer. Many studies have shown the capacity of ECM to drive disease progression towards malignancy (*27, 28*). The composition of the extracellular matrix is a prominent indicator of clinical prognosis. For example, in case of breast cancer, extracellular matrix of tumor with high expression of protease inhibitors is associated with good prognosis, while tumors with high expression of integrins and matrix metalloproteinases (MMPs) correlate with poor prognosis and risk of recurrence (*29, 30*).

(b) Soluble components: The secretary components are one of the most important functional constituents of tumor microenvironment. These components include cytokines, chemokines and polypeptide growth factors, small molecules that may be derived from either neoplastic or infiltrating immune cells. The profile of these factors is quite dynamic in nature and the balance decides the fate of cancer. Various pro and anti-inflammatory cytokines and chemokines like IL-6, TGFβ, GM-CSF, TNFα, IL-10, IL-17, small lipid molecules like prostaglandins, growth factors like epidermal growth factors (EGF), vascular endothelial growth factor (VEGF) as well as enzymes like matrix metalloproteinase (MMP) are part of soluble component of tumor microenvironment and they facilitate the growth of neoplastic cells and their survival against immune response (*31*).

(c) Cells of mesenchymal origin: These are cells derived from the mesenchyme and include fibroblasts, myofibroblasts, mesenchymal stem cells (MSCs), adipocytes and endothelial cells. While myofibroblasts and MSCs play an important role in tumor progression by releasing many pro tumorigenic factors in microenvironment, the role of

endothelial cells and pericytes are highly crucial in angiogenesis. Angiogenesis is considered as hallmark of cancer without which tumors would succumb to dormancy. Vascular endothelial cells form tight adhesions to ensure vessel integrity and pericytes covers the vessel from inside and dictate vessel maturity (*25, 27*).

(d) Cells of hematopoietic origin: This class includes different kinds of immune cells which have infiltrated the tumor and surrounding area including cells of the lymphoid lineage like T cells, B cells and natural killer (NK) cells, and those of the myeloid lineage, which includes macrophages, neutrophils and dendritic cells. All the constituent cell types either play pro-tumorigenic or anti-tumorigenic role depending upon their interaction with each other and many other factors in the microenvironment.

1.3 Theory of immunosurveillance:

1.3.1 Introduction to immunosurveillance: It is the detection and destruction of nascent cancers by the innate and adaptive immune system (*32*). Estimates of spontaneous mutations have been carried out in human diploid lymphoblast cell line, TK6, heterozygous for thymidine kinase and containing one copy of hypoxanthine-guanine phosphoribosyltransferase or coat color loci in mice. These studies revealed mutation rates of 2.0×10^{-7} mutations/gene/division or $2 - 40 \times 10^{-6}$ mutations/gamete/gene respectively (*33*). However, this does not result in an "overwhelming frequency" of carcinomas probably due to the repression of potentially transformed cells by the immunosurveillance system as first proposed by Paul Ehrlich (*34, 35*). However the idea did not get momentum until late 20th century. The basis

behind immunosurveillance system, is the ability to distinguish between the body's own cells (‘self’) and mutant phenotype of tumor cells (non-self). As the tumor cells are developed from the host's own cells they have to produce signals that are not expressed by normal cells. Most of the cancer cells expressed such signals in order to get indispensable growth advantage against normal cells. These signals are often termed as ‘tumor specific antigens’. For example majority of the cancer cells showed altered glycosylation pattern which give advantage to cancer cells in migration and metastasis (*36, 37*). This pattern can be recognized by antigen presenting cells. Though it is difficult to confirm cancer immunosurveillance hypothesis directly, there are several evidences in support of this. 1) Primary immuno-deficiency in humans and mice is associated with higher cancer risk. 2) Organ transplant recipients who are under immunosuppressive treatment are more prone to cancer 3) Immunosuppression induced by human immunodeficiency virus leads to increased risk of cancer 4) Adaptive immune system can recognize mutant tumor antigens 5) Cancer cells accumulate mutations to evade immune system 6) Immune cells can detect and eliminate pre-malignant cells and can serve as prognostic marker (*34*). Statistical analyses of cancer patients have also shown a positive correlation between the presence of lymphocytes in a tumor and increased patient survival. In one such study for melanoma, patients were categorized into brisk, non-brisk and absent based on tumor infiltrating lymphocytes (TIL) and after 5-10 years of survival monitoring, it was observed that patient with brisk TIL survived 2 to 3 times more than absent TIL while non-brisk TIL group had an intermediate survival rate (*38*).

1.3.2 Cancer immunoediting: Tumor generated from immunocompetent mice grew in both immunocompetent and immunocompromised mice of the same strain upon transplantation. However tumor from immunocompromised mice failed to grow in immunocompetent host which led to the cancer immunoediting hypothesis which proposed that the immune system not only protects host against tumor but also helped in sculpting of tumor (*39*). Cancer immunoediting involves three processes. (i) elimination (ii) equilibrium and (iii) escape, which are known as three 'E' of immunoediting (Fig 3).

(a) **Elimination**: This phase essentially involves the process of immunosurveillance where the nascent cancer cells that have developed due to failed intrinsic tumor suppressor mechanisms are detected and eliminated. When the growing tumor crosses a certain threshold, it might initiate an inflammatory response which attracts immune cells (NK cells, NKT cells, macrophages, dendritic cells) to the site, which recognize tumor antigens or tumor associated antigens and are activated. This leads to the release of pro-inflammatory cytokines like IFNγ and chemokines like CXCL9, CXCL10 which further induce death of cancer cells. This facilitates the recruitment of more number of macrophages, dendritic cells (DC) and NKT cells to the site. DC process these apoptotic cells and presents the tumor antigens to $CD4^+$ T helper cells (T_{H1}) present in the adjoining lymph nodes. This ultimately results in tumor specific cytotoxic T cell response which destroys the remaining tumor cells.

(b) **Equilibrium**: Despite the destruction of majority of tumor cells, the continuous pressure due to IFN secretion and immune activation leads to a selection

process, where some mutated cells may survive the immune destruction due to the dynamic nature of genetic mutation. In this phase, these tumor cells either remain dormant or continue to evolve, accumulating further changes. The enormous plasticity of the cancer cell genome is thought to arise from several types of genetic instability including chromosomal instability. Thus, the tumor cell's constant genomic metamorphosis may finally give rise to new tumor cell variants that display reduced immunogenicity which will provide resistance against immune attack or will hide the tumor from further immune detection.

(c) **Escape**: During this phase, the edited tumor cells become insensitive to the immune response and tumor growth proceeds unrestrained by immune pressure. In addition, the tumors skew the immune response to favor its growth. Many factors in the tumor microenvironment contribute to tumor escape such as loss of tumor antigen or downregulation of MHC molecules, soluble suppressive factors, anergy or active inhibition of T cell responses, resistance to cytotoxic pathways, overexpression of the antiapoptotic molecules and expansion of immunosuppressive cell populations (*32*).

Immune cells including lymphocytes and antigen presenting cells participate in immune editing process and play different roles.

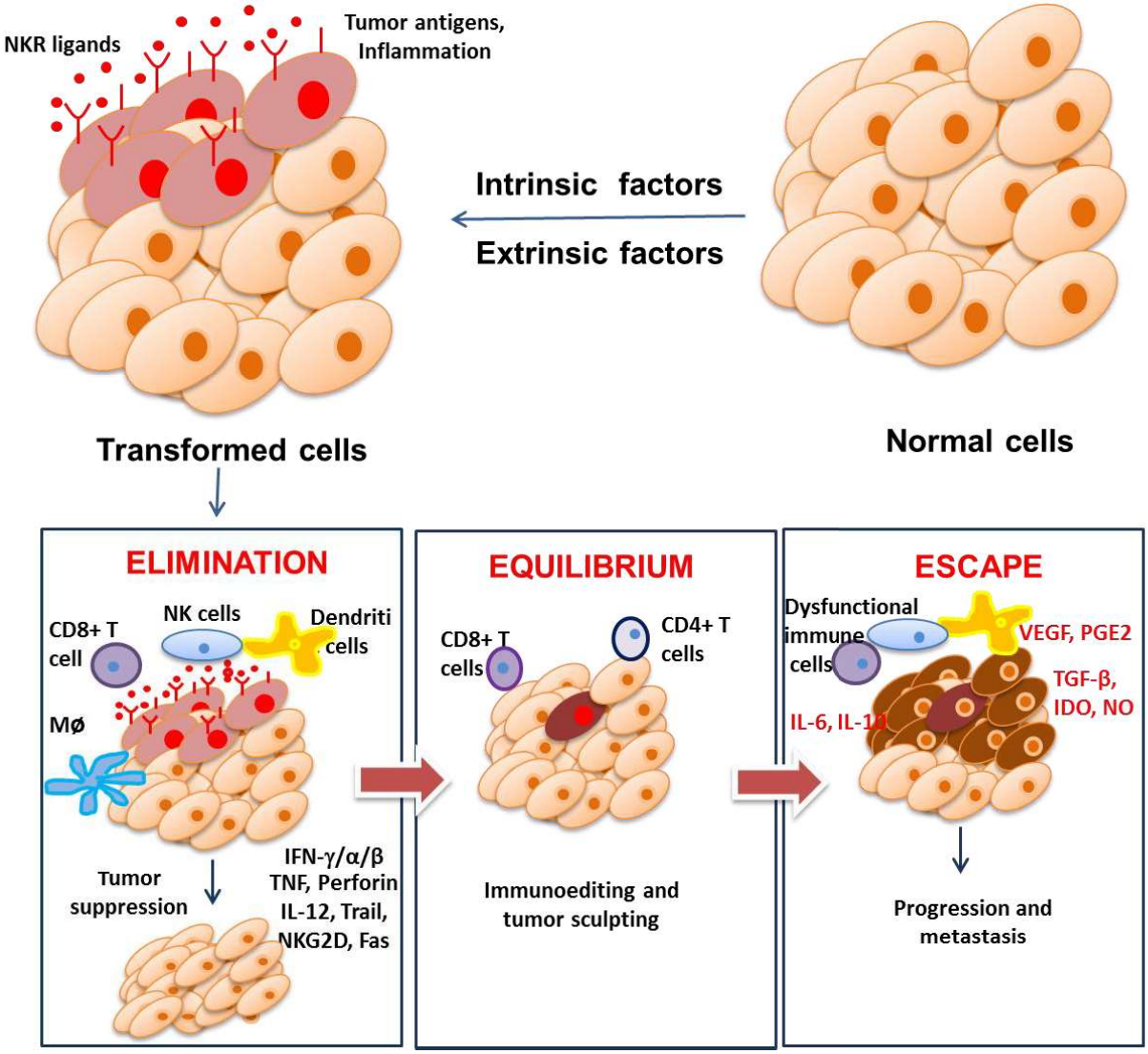


Fig 3: The three 'E' phases of cancer immunoediting hypothesis. Normal cells undergo transformation due to various intrinsic and extrinsic factors. Such phenotypes are detected by competent immune cells in elimination phase and promptly killed. However, if they escape immune detection and destruction, they are held in a state of immune dormancy in the equilibrium phase. Dormant transformed cells can undergo immunoediting, and enter into the escape phase, where they grow progressively under immunosupressive environment (Modified from *Dunn et al (32)*).

1.3.3 Cellular components of immunoediting system:

1.3.3 (a) Lymphocytes (T cells, B cells and NK cells)

(i) T cells: T lymphocytes play a central role in immunoediting of cancer. Increased incidence of spontaneous tumors in recombination-activating gene (Rag)-deficient mice or when T lymphocytes were depleted using antibodies, (*40*) confirmed the role of T lymphocytes in elimination stage of immunoediting. In addition, the tumor generated from T lymphocyte deficient mice were more immunogenic than tumor generated in wild type mice which further proved the role of T cells in tumor sculpting and thus maintaining the equilibrium stage (*41*) Conventionally, $CD8^+$ T cells are considered as the main effector cells for anti-tumor immunity. However, the activation and clonal propagation of anti-tumor $CD8^+$ T cells are dependent on dendritic cells and $CD4^+$ T cells. Tumor antigen (TA) loaded migratory DCs present TAs to naive $CD4^+$ T cells that further helps in the clonal expansion of TA-specific $CD8^+$ T cells. Tumor antigen-specific $CD4^+$ and $CD8^+$ T cells migrate to the primary tumor site, where the CTLs target the remaining TA-expressing tumor cells (*42*). Dendritic cells can directly activate TA specific $CD8^+$ T cell response through cross-presentation also, where TAs are presented to CTL through MHC I molecules (*43*). Role of $CD4^+$ T cells in cancer immunoediting is also crucial at several levels. $CD4^+$ T cells help, by means of temporal release of IL-2 is required for the optimal induction and clonal expansion of cytotoxic $CD8^+$ T cells (*44*). $CD4^+$ T cells also play a pivotal role in the generation and maintenance of functional and long-lived $CD8^+$ memory T cells (*45*). Recruitment of a specialized subset of $CD4^+$ T cells, $CD4^+CD25^+$

regulatory T cells (Treg), into tumors has been proposed to be one of the major tumor immune escape mechanisms. Treg cells hamper the functions of $CD8^+$ T cells and natural killer cells, induce T cell tolerance and suppress immune response (*46, 47*). Another subset of T cells known as γδ T cells exhibit important roles in immune-surveillance and immune defense against tumors. γδ T cells mediate anti-tumor response mainly by secreting pro-apoptotic molecules and variety of chemokines and cytokines, such as IFNγ, perforin-granzyme, tumor necrosis factor (TNF), and participating in TNF-related apoptosis-inducing ligand (TRAIL)/TRAIL receptor (TRAILR) mediated killing (*48, 49*).

(ii) B cells: B cells display variable pro- and anti-tumor activities deriving from their functional plasticity and phenotypic heterogeneity. As the producers of antibodies, B cells mediate the humoral immune response against cancer. In addition, there is accumulating evidence that B-cell function is relevant to carcinogenesis. Cancer patients often develop specific antibody responses against tumor antigens, which are mostly correlated with poor survival. Through the secretion of IL-10 and TNFα, B cells may manipulate myeloid cell differentiation and function and thereby accelerate tumor progression (*50*). Studies have also shown a positive correlation between higher numbers of total $CD20^+$ B cells and higher tumor grade (*51*).

(iii) NK cells: Natural killer cells represent one of the three subsets of lymphocytes besides T and B cells but belong to the innate immune system of the body. They are unique because of their ability to directly target and kill tumor cells. Unlike T cells, NK cell recognition is not governed by antigen specificity of target cells but

is mediated by the signals delivered through several activating and inhibitory receptors. The balance between activating and inhibitory signals decides the response of NK cells. They can be activated due to lack of inhibitory signals leading to lysis of the host cell. NK cell activation also involves distinction between self and non-self. One such mechanism involves MHC class I-dependent recognition mode in which virally infected or malignant cells with an impaired MHC class I expression are attacked by NK cells, whereas 'healthy' autologous cells are protected from NK cytotoxicity (*52*). Natural killer cells perform their cytotoxic activity through granzyme B- and perforin-mediated apoptosis or by expression of death receptor ligands such as FasL and TNF-related apoptosis-inducing ligand (TRAIL) (*53-55*). NK cells are one of the most important components of immunosurveillance mechanism. Evidence supporting this came from experiments where it was shown that frequencies of spontaneously arising tumors or tumors induced by the chemical carcinogen methylcholanthrene (MCA) were higher in mice that were genetically deficient for key effector molecules of NK cells or the respective receptors (*40*). NK cells not only directly target tumor cells by means of cytolysis or IFNγ secretion, but may also indirectly contribute to tumor control by inducing an efficient T-cell-mediated anti-tumor response. IFNγ released by NK cells are the key mediator of the elimination process whereby they not only kill the cancer cells but also instrumental in the stimulation and maturation of dendritic cell (DC) to a IL-12-producing phenotype to promote an anti-tumor $CD8^+$ T-cell response (*56*).

1.3.3 (b) Antigen presenting cells (macrophages and dendritic cells)

(i) Macrophages: Macrophages are among the most important phagocytic cells which also play the role of professional antigen presenting cells. They mediate their effects not only through phagocytosis but also through the production of various soluble factors such as cytokines and chemokines. Due to their immune surveillance role, macrophages sense a wide spectrum of stimuli, like viral, microbial and parasite antigens, immune complexes and apoptotic or necrotic cells to various mediators released by other cells. The primary function of macrophages is the defense of the body against pathogens as a part of innate immune system. They also play an important role in both the initiation and resolution of inflammation. Moreover, macrophages can exhibit different responses depending on the type of stimuli they receive from the surrounding microenvironment, varying from pro-inflammatory to anti-inflammatory (*57, 58*). Two major macrophage phenotypes have been proposed: M1 and M2, which exhibit a distinct range of responses. M1 macrophages, are the classically activated macrophages which are highly phagocytic, produce large amounts of reactive oxygen species and promote a T_H1 response. M1 macrophages contribute towards generation of inflammation and play crucial role in identification of tumor-associated antigens and subsequent destruction of tumor cells and their presence usually indicates good prognosis (*59, 60*). M2 macrophages are anti-inflammatory, produce IL-10, upregulate production of IL-4 and other anti-inflammatory cytokines, promote a T_H2 response and aid in the process of

angiogenesis and tissue repair (*61*). However, in the context of cancer, these tissue repair and wound healing pathways of macrophages are co-opted by the tumor cells for its own growth and hence becomes pro-tumorigenic. IL-4 is an important cytokine in the healing process because it contributes to the production of the extracellular matrix (*62*). Tumor microenvironment plays a significant role in deciding the polarity of macrophages which express high plasticity and flexibility in phenotypes and can, to some extent, be reversed *in vitro* and *in vivo* (*63*).

(ii) **Dendritic cells:** Professional antigen presenting cells (APC) are important for initiation of immune response against pathogens like bacteria, virus and crucial for anti-tumor immunity. Among all APC, dendritic cells are considered to be most potent because of their unique ability to activate both $CD4^+$ and $CD8^+$ T cells against exogenous antigens (*64*). This ability is particularly very important in generating immune response against tumor. Apart from its role in generating immune response, DC also play a very important role in generation of central and peripheral tolerance (*65*). Among the three professional antigens presenting cells (macrophages, B cells and dendritic cells), dendritic cells have been shown to be the most effective in generating immune response. Steinman et al showed that dendritic cells enriched population was 30-40 fold more potent in generating proliferation of allogenic T cells compare to macrophages and B cells enriched population in in a mixed leucocyte reaction (*66*). It was further shown that elimination of DC by using DC specific 33D1 monoclonal antibody reduced the MLR stimulating

capacity of splenic adherent cells by 75-90%, similar reduction was not seen with removal of macrophages and B cells . These experiments concluded that other APC do express alloantigens like Ia and H-2 antigens but DC were the most critical accessory cells required for the induction of lymphocyte response owing to much higher expression of MHC II molecules.

1.4 Immunobiology of dendritic cells:

1.4.1 History of dendritic cell research: Dendritic cells were first discovered by Ralph Steinman and the Zanvil A. Cohn in 1973 (*66*). Though, it was recognized that apart from lymphocytes, other assisting cells are required for the development of immunity, they were not characterized. Steinman came across a novel cell type in murine spleen, which had unusual tree-like or "dendritic" processes and therefore named them dendritic cells. These cells were distinct from macrophages in phenotype and did not easily mediate endocytosis, a characteristic feature of macrophages. Also unlike macrophages, the dendritic cells detached from culture surfaces, had poor viability, had few digestive bodies or lysosomes, lacked the key receptors for antibody-coated particles (Fc receptors), and were poorly phagocytic *in vivo* and *in vitro*. At the same time, Veerman proposed the presence of “interdigitating cells” in the rat spleen which induced T cells to differentiate and proliferate (*67*). Later studies revealed DC’s potent stimulatory role in immune function, especially activation of T cells. They were found in many organs of several animal species, including human blood. Subsets of dendritic cells were identified, each having its own surface markers. Dendritic cells were seen in the T-cell areas of lymph nodes, the ideal location for initiating immunity.

1.4.2 DC lineage and subset: DC originates from haematopoietic stem cells and can have two types of origin, either lymphoid or myeloid based on type of precursors cells (*68-70*).

1.4.2 (a) Plasmacytoid DC: Lymphoid DC are called as plasmacytoid DC (pDCs) while myeloid lineage DCs are considered as the "classical" DC (cDC) (Fig 4). Plasmacytoid DC lack most myeloid markers and have growth requirements distinct from those of cDCs. They express low levels of major histocompatibility complex class II (MHC-II) as well as costimulatory molecules and are $B220^{+}$ and $CD11c^{int}$. pDC are uniquely able to produce large amounts of the antiviral cytokine IFNα and initiate T cell immunity to viral antigens. pDC are considered poor presenters of exogenous Ag, but can present endogenous Ag (*71*).

1.4.2 (b) Classical DC (cDC): cDC are the most studied DC population and can be divided into resident DC and migratory DC (*72*).

Resident DCs are MHC $II^{int}CD11c^{hi}$ and can be divided into three subsets: $CD4^{+}CD8^{-}$, $CD4^{-}CD8^{+}$, and $CD4^{-}CD8^{-}$ (double-negative) DCs. The main function of $CD8^{+}$ cDC is cross-presentation of exogenous antigens to $CD8^{+}$ T cells, whereas $CD8^{-}$ cDC are involved in activating $CD4^{+}$ T cells. The $CD4^{+}$ and the double-negative resident DCs are also $CD11b^{+}$. Lymphoid tissue–resident DC are present in all lymphoid organs, thymus, spleen and lymph node (1). Mouse thymus contains $CD11c^{int}CD45RA^{+}$ pDCs and two $CD11c^{hi}CD45RA^{-}$ cDC subsets that are segregated based on CD8α and the signal regulatory protein-α (Sirpα) expression, as $CD8\alpha^{+}Sirp\alpha^{-}$ and $CD8\alpha^{-}$/loSirpα^{+} cDC subsets. Although thymic cDC share

many common features with other peripheral DC subsets, they mostly present self-antigens (Ag) rather than foreign Ag (2, *73*). Spleen is a rich source of lymphoid tissue-resident DCs. Three cDC subsets have been identified in the mouse spleen based on the surface expression of CD8α and CD4, in addition to high levels of CD11c expression on all cDCs. These cDC subsets are $CD11c^{hi}CD4^{-}CD8\alpha^{+}$, $CD11c^{hi}CD4^{-}CD8\alpha^{-}$, and $CD11c^{hi}CD4^{+}CD8\alpha^{-}$. The $CD4^{-}CD8\alpha^{+}$ cDCs also express CD205, but not Sirpα. In contrast, both $CD4^{-}CD8\alpha^{-}$ and $CD4^{+}CD8\alpha^{-}$ cDC subsets do not express $CD205^{-}$, but are Sirp-α+, and are sometimes considered as one $CD8\alpha^{-}$ cDC subset. In addition to cDCs, pDCs are also found in mouse spleen. They are defined as $CD11c^{int}CD45RA^{+}B220^{+}SiglecH^{+}$. DCs in the spleen develop from precursors that circulate in the blood and develop into immature $CD8\alpha^{-}$ and $CD8\alpha^{+}$ DC subsets after entering the spleen. (3) The DC populations found in mouse LNs are more complex. In addition to the three phenotypically and functionally equivalent cDC populations found in mouse spleen, two additional subpopulations have been described in the skin draining LNs. These correspond to the mature $CD8\alpha^{lo}CD205^{int}$ and $CD8\alpha^{lo}CD205^{hi}$ cDC that migrate from the epidermis and dermis, respectively, to the LNs (*74*). DCs found in the lymph nodes (LN) are thought to travel there through the afferent lymph from non-lymphoid tissues. Some DCs and DC precursors are also thought to reach LN across the high endothelial venules.

Non-lymphoid tissue-resident DCs are present in most tissues in the steady state and express the hematopoietic marker CD45, the integrins CD11c and CD103 and major histocompatibility complex class II (MHCII) (*75*). Studies of non-lymphoid

tissue DCs so far have largely involved tissues that are in contact with the body surfaces, such as the skin, lung, and intestine. Intestinal DC ($MHCII^{+}CD11c^{+}CD64^{-}$) subsets include $CD103^{+}CD11b^{-}$ and $CD103^{+}CD11b^{+}$ (*69, 76*). The lung is also vulnerable to pathogenic assault and therefore the conducting airways are lined with an intraepithelial highly dendritic network of $MHCII^{hi}CD11c^{hi}$ cells that are mostly $CD11b^{-}$ and express langerin and the mucosal integrin CD103 (αEβ7). These $CD103^{+}$ cDC are involved in cross-presentation of antigens to cytotoxic $CD8^{+}$ T cells. The lamina propria of the conducting airways contain $MHCII^{hi}CD11c^{hi}$ cells that express high levels of $CD11b^{+}$ $D103^{-}$ Sirp-α^{+} which are a rich source of proinflammatory chemokines. In the skin, DCs may include pDC, cDC, and moDC. Under steady-state conditions, pDCs are absent from the skin and have been observed in inflamed skin where they promote wound repair. Epidermal DCs are also known as Langerhans cells (LCs). The LC population is maintained by local precursor cells in the steady state. The most abundant type of DC in healthy dermis are the dermal $CD11b^{+}$ cDCs. There are $CD11c^{+}CD11b^{-}$ cDCs which express the C-type lectin langerin (also known as CD207 and CLEC4K) and include $CD103^{+}$ and $CD103^{-}$ cells (*77*).

Migratory DC develop in peripheral tissues, such as the skin and the gut, and migrate constitutively into lymph nodes. Migratory DCs include various subsets that can be divided largely based on langerin, CD11b, and CD103 expression.

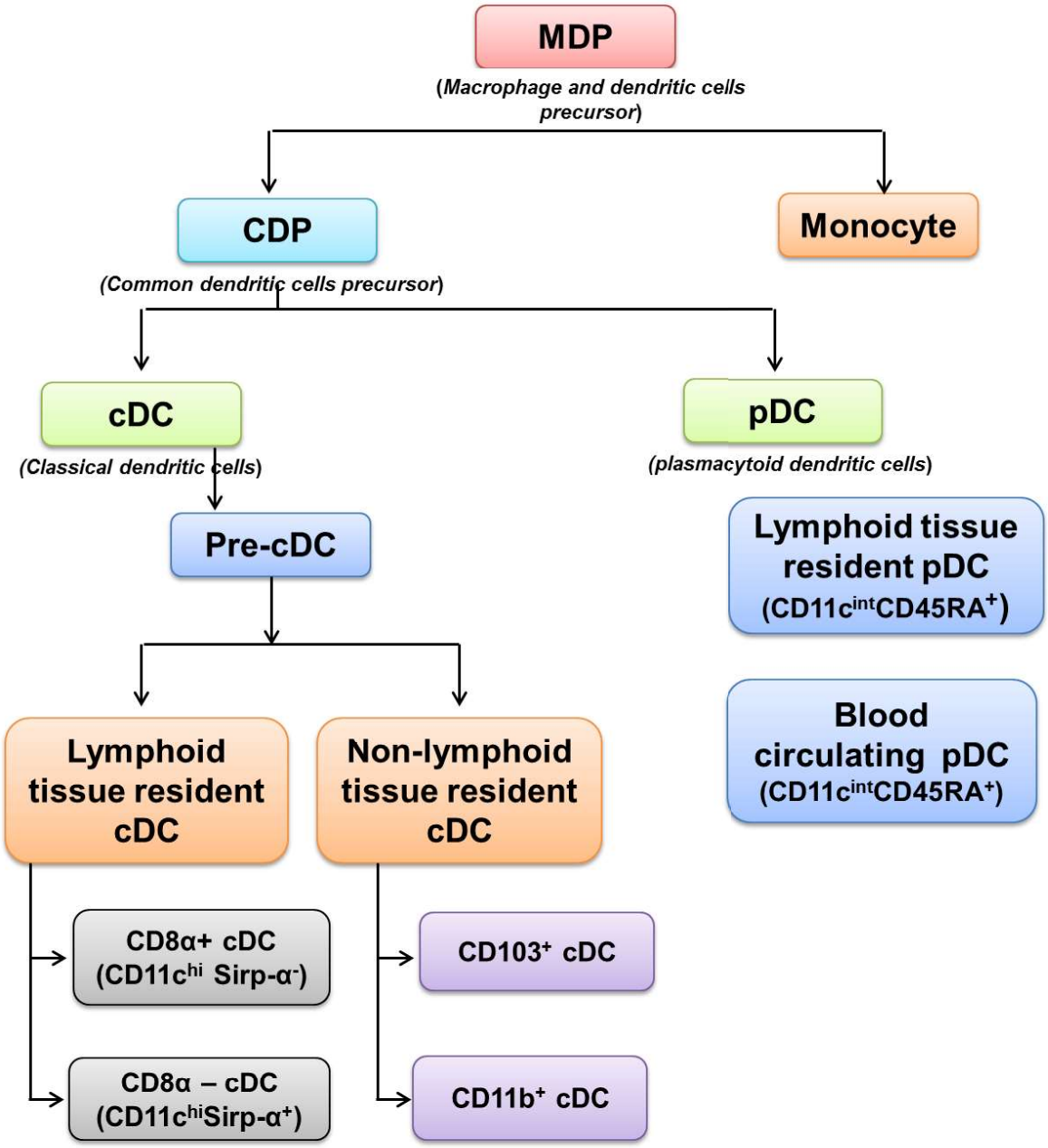


Fig 4: Schematic representation of different lineages of dendritic cell. This is based on their location and phenotypes derived from a common macrophage and dendritic cell precursor (MDP).

1.4.3 Function of dendritic cells:

Dendritic cells are the professional antigen presenting cells. They play diverse roles linking innate and adaptive immune response. However, all their functions are based on the ability to sense danger signals through pattern recognition receptors (Fig 5). cDC constitutes the major proportion of DC population (*78*). Both the lymphoid tissue resident $CD8^+$ DC and non-lymphoid tissue resident $CD103^+$ DC have similar origin and also share similar functional attributes. $CD103^+$ cDC are specifically located in non-lymphoid tissues at the interface with the environment and migrate to T cell zone of the draining LN once they are loaded with antigens (*79*). $CD8^+$ cDC are in the marginal zone of spleen mainly where they encounter the blood antigens and then migrate to T cell enriched regions.

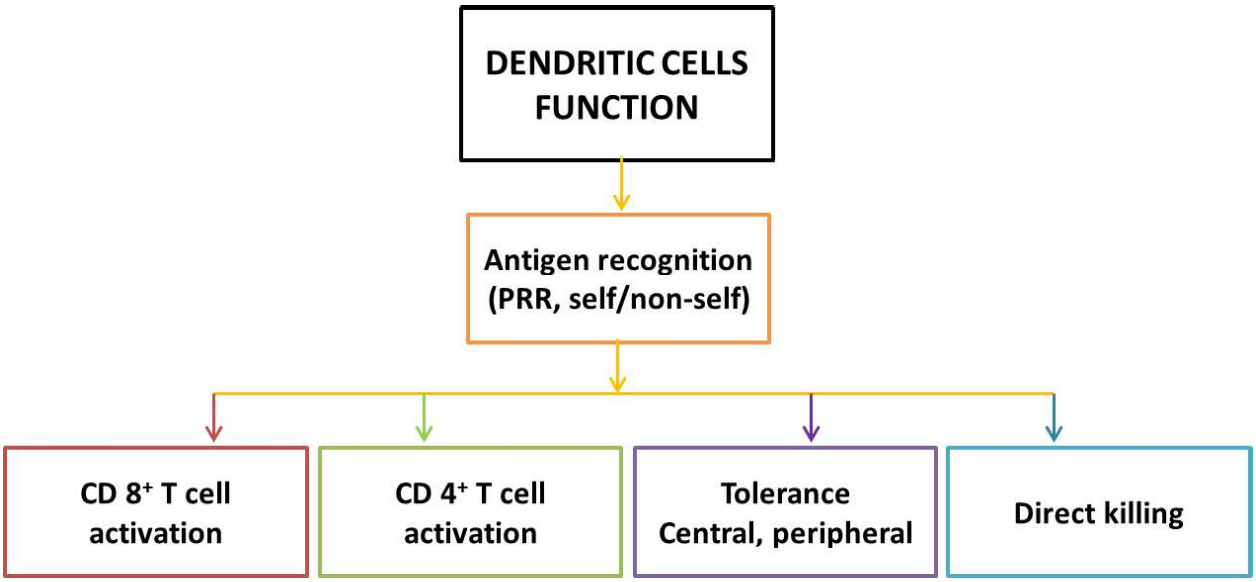


Fig 5: Schematic representation of different functions of dendritic cells. Dendritic cells link the function of innate and adaptive immune system by recognition of antigens through pattern recognition receptors (PRRs).

1.4.3 (a) Antigen recognition: Dendritic cells express variety of pattern recognition receptors (PRR) which recognise highly conserved structures of pathogens called pathogen-associated molecular patterns (PAMPs) that are distinct from the host (*80*). These PRR include toll-like receptors (TLRs), retinoic-acid-inducible gene (RIG)-like helicases (RLHs), or nucleotide-binding domain and leucine-rich repeat-containing molecules (NLRs). There is a total of 10 human and 12 mouse TLRs. Lipopeptides and other components of Gram-positive bacterial cells activate TLR2 in conjunction with either TLR1 or TLR6; lipopolysaccharides from bacterial cell walls is detected by TLR4 resulting in cellular responses leading to the expression of inflammatory genes (*81*). Flagellin is detected by TLR5; poly I:C, a double-stranded RNA (dsRNA) analog, is detected by TLR3; unmethylated DNA and CpG-oligodeoxynucleotides (CpG-DNA) are detected by TLR9; and single-stranded RNA and its synthetic analogs resiquimod, imiquimod, and loxoribine activate TLR7. The ligands for TLR8, TLR10 are only present in humans and those for TLR11–13 are only present in mice and are not known (*82*). As both the **l**ymphoid tissue $CD8^+$ DCs and nonlymphoid tissue $CD103^+$ DC have similar functional attributes, both cDC types express a similar TLR, C-type lectin receptor, and chemokine receptor profile. Both express high levels of the dead cell binding scavenger receptor CD36, high level of C-type lectin Clec9A (sense necrotic bodies) as well as DEC205 (*83, 84*). $CD11b^+$ cDC are potent cytokine producers in the steady state and express cytoplasmic viral sensor receptors RIG-I and melanoma differentiation antigen 5 (MDA5) with expression of different PRRs as compared to $CD8^+$ and $CD103^+$ cDC. (*85*).

1.4.3 (b) $CD8^+$ T cell activation: Among all cDC, $CD8^+$ and $CD103^+$ cDC are specialized for their role in presenting microbial and cell-associated antigens to $CD8^+$ T cells (*86, 87*) and this is highlighted by the inability to elicit an effective virus specific cytotoxic T cell response and inability to reject fibrosarcoma tumors in mice deficient in $CD8^+$ and $CD103^+$ DC (*88*). The key feature of cDC which separates them from other antigen processing cells is their ability to cross present exogenous antigens to $CD8^+$ T cells through MHC I molecules (*89*). This function requires two important aspects, a low degrading capacity of endocytic pathways and a mechanism to transport antigens from endosome to cytoplasm (*90*). The phagosomes of $CD8^+$ cDCs are less stable due to overexpression of adipose differentiation related protein which causes oxidative stress and destabilise phagosome membrane and facilitate release of endosomal antigens to cytoplasm. Similarly Rac2, a GTPase that maintain a less acidic environment in phagosome, limits its endocytic function (*91*). Also, $CD8^+$ cDCs express more MHC-I related genes than $CD11b^+$ cDC. Though $CD11b^+$ cDC are not classically considered to be involved in cross presentation, there are some reports which suggest that cross presentation can be induced in $CD11b^+$ cell by ligation of the Fcγ receptor (*92*).

1.4.3 (c) $CD4^+$ T cell activation: $CD11b^+$ cDC are considered to be more efficient in MHC-II presentation than $CD8^+$ and $cCD103^+$ DC (*93*). They express higher level of genes coding for proteins involved in the MHC-II antigenic pathway. While $CD8^+$ splenic cDC mainly produce T_H1 polarizing cytokine IL-12, dermal $CD103^+$ cDC control the induction of pathogen-specific $CD4^+IFN\gamma^+$ T cells upon cutaneous infection.

1.4.3 (d) Central and peripheral tolerance: Dendritic cells are very critical for maintenance of both central and peripheral tolerance. During the development of T cells in the thymus, both $CD8^+$ cDCs and $CD11b^+$ cDC are involved in negative selection where they participate in eliminating those cells bearing "self-reactive" antigens through induction of regulatory T cells (Tregs), anergy or apoptosis (*65*) This process constitutes the process of central tolerance. In the periphery, $CD8^+$ and $CD103^+$ cDCs are thought to participate in deletional tolerance of self-reactive T cells and the induction of antigen-specific Tregs. The steady-state migrating DCs are loaded with tissue antigens, probably internalized by phagocytosis of apoptotic cells in the tissues or intestine which are transported to the regional lymph node for tolerance induction (*94*).

1.4.3 (e) Direct killing: Recent studies have indicated a primordial role of dendritic cells as effector cells that are tumoricidal in nature. This phenotype of DC is named as 'Killer' DC (KDC), which not only recognise target cells but can also generate antigen-specific anti-tumor T-cell responses *in vivo (95-97)*. Various mechanisms have been postulated for killer DC phenotypes such as Fas or TNFα-dependent apoptosis (*98, 99*). However, the origin and lineage of killer DC is a matter of debate and needs to be explored further.

1.4.4 Cancer immunotherapy-

DC immunotherapy boosts the immune system to fight malignancy unlike chemotherapy and radiotherapy which exhibit direct cytotoxicity against cancer cells. These approaches include (i) cell based therapies where DCs, T cells or NK cells are directly transferred into patient's body (*100*), (2) treatment with antibodies targeting members of the tumor necrosis factor receptor superfamily or tumor antigens (*101*), (3) administration of oncolytic viruses (OVs) for the preferential killing of cancer cells (*102*), (4) treatment with checkpoint inhibitor to relieve immunosupressive signals (*103*). Owing to its crucial role in anti-cancer immune response, dendritic cells are important components of many cancer immunotherapy protocols.

1.4.4 (a) DC based cancer immunotherapy: Sipuleucel-T (Provenge) was the first FDA approved dendritic cell vaccine against cancer that was given U.S. Food and Drug Administration (FDA) approval in 2010 to treat advanced prostate cancer (*104*). Sipuleucel-T is an active cellular immunotherapy, in which autologous peripheral-blood mononuclear cells (PBMCs), including dendritic cells are extracted through a leukopheresis procedure. DC are further activated *ex vivo* with a recombinant fusion protein (PA2024). PA2024 consists of two parts, a prostatic acid phosphatase antigens which is expressed by majority of prostatic cancer cells and granulocyte-macrophage colony-stimulating factor, an immune-cell activator and then re-infused into the patient (*105*). A double-blind, placebo-controlled, phase 3 trial was conducted with 521 patients. Total 341 patients were randomly assigned Sipuleucel-T while 171 patients were on placebo control. The trials showed significant prolonged overall survival among men with metastatic

castration-resistant prostate cancer with the use of Sipuleucel-T (*104*). Apart from Sipuleucel-T more than 100 phase I–III clinical trials based on dendritic cells immunotherapy are going on against prostate cancer, melanoma, renal cell carcinoma, in glioma (*106*). Owing to DC limitation as monotherapy for cancer treatment, it has been tried in combination with various chemotherapies (*107*). DC vaccination was also tested with the addition of a COX-2 inhibitor (Celecoxib) and chemotherapeutic agent cyclophosphamide in melanoma in a phase III trial and it has shown longer overall survival compared with outcomes from chemotherapy alone (*108*).

1.4.4 (b) Checkpoint therapy: Apart from DC based cancer immunotherapy checkpoint inhibitors have been approved by FDA (*109*). Checkpoints are the T cell receptors which are involved in immune regulation by inhibitory signals. Cytotoxic T lymphocyte antigen-4 (CTLA-4) and programmed death-1 (PD-1) transmit inhibitory signals when bound to their ligands, B7.1/B7.2 and PD-L1 respectively which are present on APCs or cancer cells. Various checkpoints inhibitors are currently been used in cancer therapies. A CTLA-4 inhibitor Ipilimumab was the first checkpoint inhibitor that has got FDA approval for treatment of unresectable metastatic melanoma in 2011 (*103*). Nivolumab and Pembrolizumab are the FDA approved PD1 inhibitor used for the treatment against solid tumors and melanomas. Atezolizumab is the PD-L1 inhibitor which got FDA approval in 2016 as a second line treatment against urolthelial carcinoma with progression on or after platinum therapy (*110*).

1.4.4 (c): CAR T-cell therapy: In another immunotherapy approach against cancer, T cells derived from patient are engineered and transferred back to patient, It is called as chimeric antigen receptor (CAR) T-cell therapy. The basic principle of this therapy is that the T cells are engineered to expressed chimeric receptors which are recombinant receptors against tumor antigens. These CAR T-cells combine antigen-binding function with specificity of an antibody and T-cell activating functions. The chimeric receptors have three parts: An extracellular ligand recognition domain (against specific cancer antigen) which is usually a single-chain variable fragment (scFv), a transmembrane linker domain that span the membrane and an intracellular signaling domain that propagate the signal for T cell activation after the extracellular domain binds with the cancer antigen. The latest third generation CAR T cells combine multiple signaling domains, such as CD3ζ -CD28-41BB or CD3ζ-CD28-OX40, to augment T cell effector functions (*111*). In 2017, Tisagenlecleucel was approved by the FDA for the treatment of relapsed or refractory B-cell acute lymphoblastic leukaemia in children (*112*). Axicabtagene ciloleucel was second CAR T-cell therapy that has got FDA approval for treatment of B cell lymphomas (*113*).

1.4.5 Transcriptional regulation of dendritic cell lineage:

The differentiation of dendritic cells into specific lineages is controlled by different transcription factors. The commitment of the progenitor cell to different subtypes of DC is decided by the presence or absence of these transcriptional factors (Fig 6).

(a) Batf3: The basic leucine zipper transcription factor ATF-like 3 (Batf3) was the first transcription factor that was shown to have a role in the development of the $CD8\alpha^+$ DC. It represses Nuclear factor of activated T cells- Activator protein 1 (NFAT–AP) activity by competing with fos for jun dimerization (*114*). Although Batf3 is expressed in all cDCs including the $CD8^+$ and $CD103^+$ cDCs and the $CD11b^+$ cDCs, $Batf3^{-/-}$ mice lack $CD103^+$ cDCs and have reduced spleen $CD8^+$ cDC, but maintain normal numbers of $CD8^+$ LN cDC (*115*).

(b) IRF8: IRF8 (IFN regulatory factors 8) plays a critical role in myeloid cell differentiation while inhibiting the development of granulocytes. *Irf8*$^{-/-}$ animals develop a myeloproliferative disease distinguished by excessive granulocyte production with a lack of pDC, spleen-resident $CD8^+$ cDC, and nonlymphoid tissue $CD103^+$ cDC (*116, 117*). Distinction between $CD8^+$ cDC and $CD8^-$ cDC is also marked by expression of IRF8 in $CD8^+$ cDC. Together with transcription factors BATF3, Id2 and mTOR, IRF8 control the development of $CD8^+$ cDC and $CD103^+$ cDC, whereas the differentiation of $CD8^-$ cDCs is controlled by the transcription factors IRF2, IRF4 and Notch2. IRF8 also plays a critical role in DC function as it controls $CD8^+$ cDC maturation and IL-12 production (*118*).

(c) **Zbtb46**: Zbtb46 (zinc finger transcription factor 46) expression is associated with the commitment of common dendritic cell precursors (CDPs) to the cDC lineage only and for this reason Zbtb46 can serve as a useful marker for distinguishing cDCs from other tissue phagocytes (*119*). It is found to be expressed in spleen $CD8^+$ and $CD11b^+$ cDC, non-lymphoid tissue resident $CD103^+$ cDC whereas it is absent in pDC, monocytes, and macrophages (*120*). Zbtb46 overexpression in bone marrow progenitor cells inhibited granulocyte potential and promoted cDC development, while in Zbtb46 deficient mice, although cDC was developed, they maintained expression of granulocyte colony-stimulating factor and leukemia inhibitory factor receptors, which are normally down-regulated in cDCs (*119, 121*).

(d) **PU.1**: It belongs to the Ets family of transcription factors. Conditional deletion of PU.1 at monocyte MDP stage of DC development showed that differentiation was abrogated in absence of PU.1 which highlighted the requirement of PU.1 for DC commitment (*122*). Also PU.1 lies upstream of Fms-related tyrosine kinase 3 (FLT3) and Granulocyte monocyte colony stimulating factor receptor (GM-CSFR) and is required for the development of DCs via both pathways (*123*).

(e) **E2-2:** E2-2 transcription factor (basic helix-loop-helix (bHLH) encoded by the gene Transcription factor 4 (*Tcf4*), is a key determinant of pDC differentiation. Germline or conditional deletion of E2-2 led to a complete loss of the pDC and abolished the ability of mice to respond to unmethylated DNA (*124*). Recent findings have suggested that E2-2 acts as a repressor to many cDC specific genes and thus

promote pDC development (*125*). Deletion of transcription factor E2-2 even from mature peripheral pDCs caused their spontaneous differentiation into cells with cDC properties including loss of pDC markers, increase in MHC class II expression and induction of cDC signature genes (*126*).

(f) **Id2:** Id2 (Inhibitor of DNA Binding Protein 2) is a class I basic helix loop helix (bHLH) transcription factors. E2-2 class of transcription factors binds with a conserved motif called E-box which determines pDC fate of dendritic cells. However DNA-binding activity of these E2-2 heterodimers can be interrupted by formation of a complex between E2-2 and members of the inhibitor of differentiation (Id) HLH protein family. Among all four members of the Id protein family, Id2 and Id3 appear to be the major inhibitors of E2 protein activity during lymphocyte development and its expression was highest in $CD8\alpha^{+}$ and $CD103^{+}CD11b^{-}$ DC though present in all cDC subsets (*76*). Loss of Id2 results in the failure of these two subsets, $CD8\alpha^{+}$ and $CD103^{+}$ DCs (*127*). DCs developing in Id2-deficient mice also show de-repression of many genes normally associated with B cells as well as pDCs, as they share a large common gene signature. The balance between E2-2 and Id2 determines the choice between cDC and pDC fates.

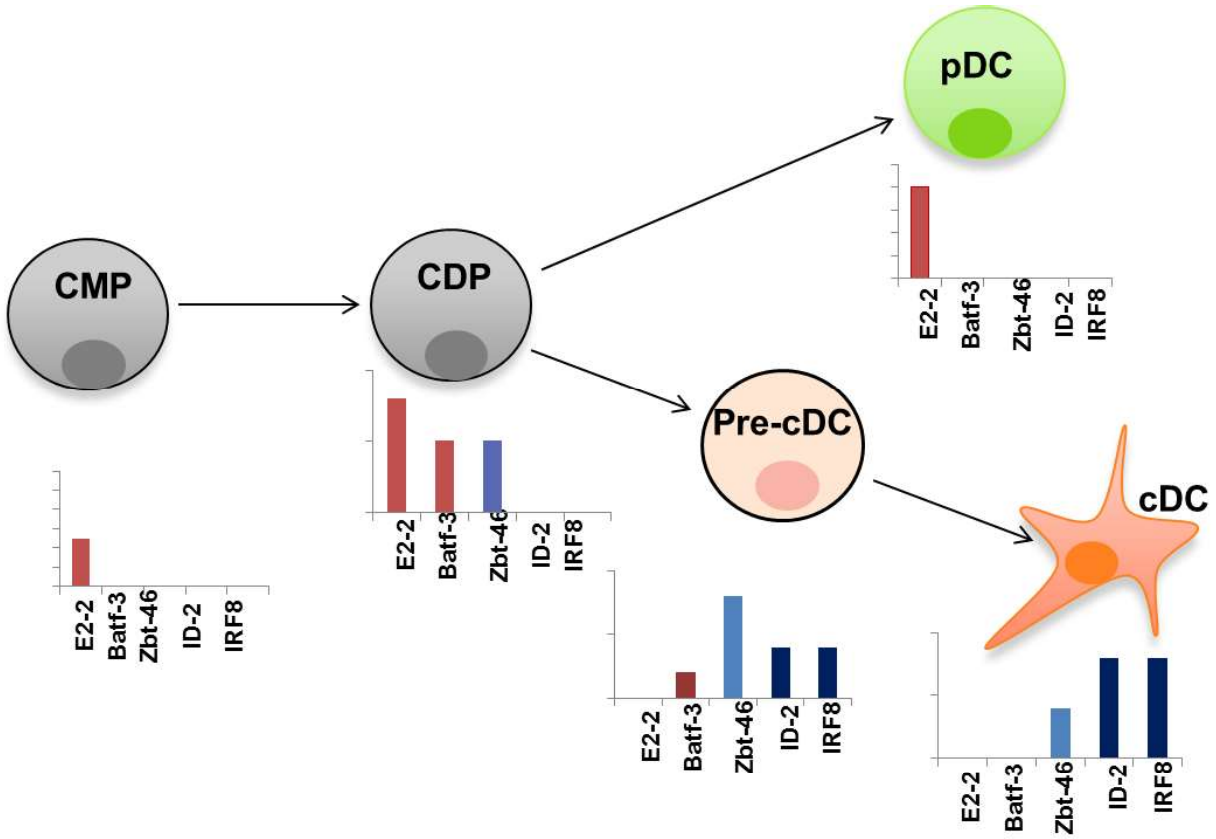


Fig 6: Schematic representation of expression of lineage specific transcription factors of dendritic cells. Several transcription factors regulate the development of different lineages of dendritic cells and serve as signature marker for that lineage. However it is the co-ordination between these transcription factors that decide the final fate of dendritic cells (*128*).

1.5 miRNA and dendritic cells:

1.5.1 Role of miRNAs in DC development: Micro RNAs (miRNAs) are endogenous small noncoding RNAs that post-transcriptionally regulate gene expression by binding to target mRNAs and inhibiting their translation through mRNA degradation or translational repression. miRNAs are generated through a specialized pathway involving the RNase,

Dicer that produces RNA duplexes of ~21 bp in length. Recent studies have shown that more than 60% of human genes have miRNA binding sites in 3' UTR region (*129*). Different cell types have different miRNA profiles which are dynamic in nature. Dendritic cells being heterogeneous in nature have been shown to be regulated by number of miRNAs. DC lineages have also shown to be regulated by various miRNAs. miRNA 126 is specifically expressed by pDC, where it controls the survival and function of pDC and regulate the expression of genes encoding molecules involved in the innate response, including *Tlr7, Tlr9 and Nfkb1 and Kdr* (*130*). Similarly miR 223 has been identified as a key regulator of differentiation and function of $CD103^+$ human cDC (*131*). However, a DC specific (CD11c-driven) knockout of Dicer showed no apparent immune phenotype, probably because most DC have short life span and the half- life of miRNA is 5 days. Only Langerhans cells which have a half-life of several weeks, showed increased apoptosis, altered surface receptor expression, and a block in the maturation process, which rendered them unable to efficiently prime $CD4^+$ T cells (*132*). Multiple miRNA expression profiling at various stages of DC development and differentiation have given rise to many overlapping miRNA as well as unique mRNA profiles. Fig 7 summarises some of the most common miRNA that have been identified at various stages of DC development.

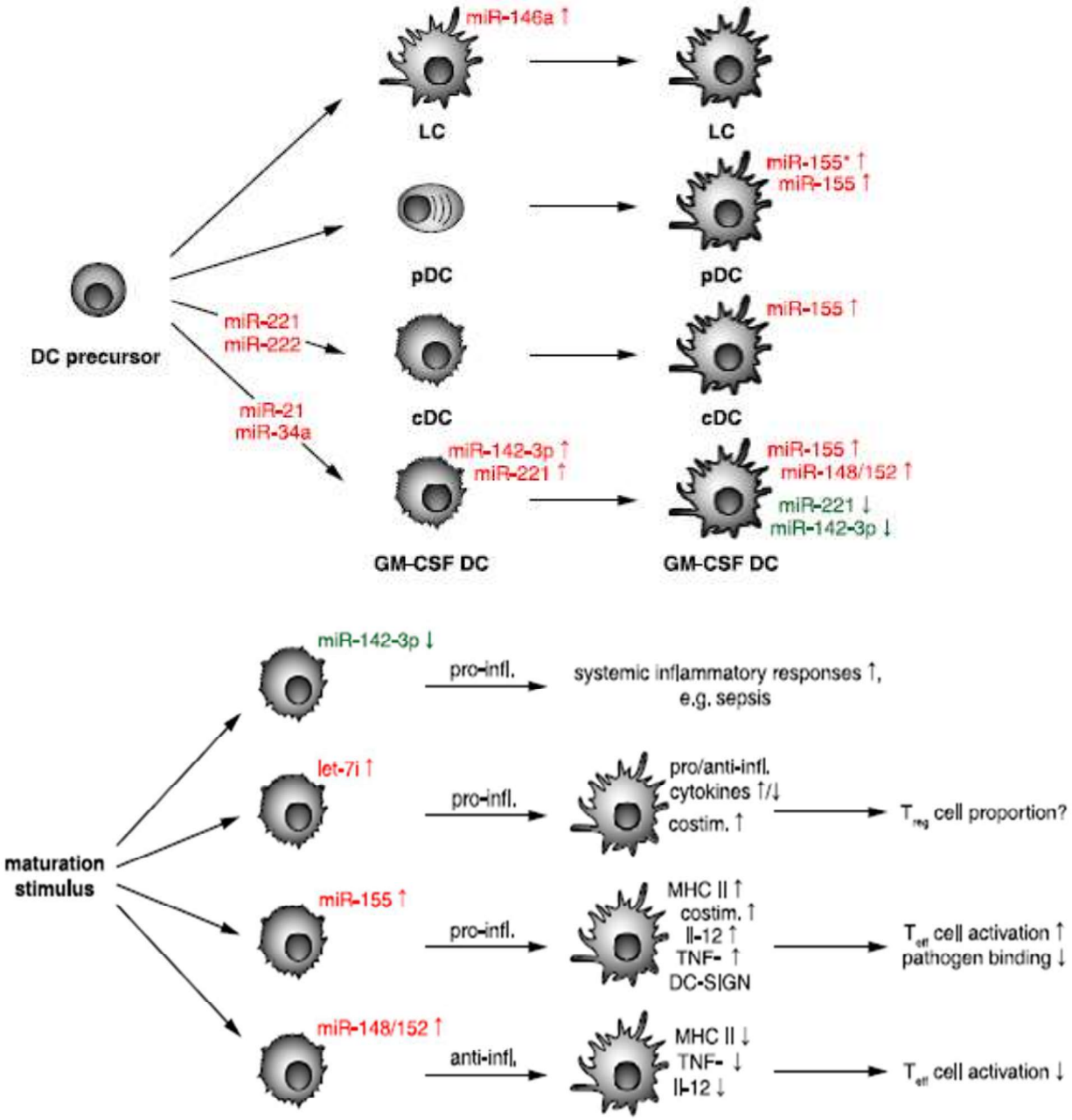


Figure 7: Schematic representation of role of miRNA at different stages of DC development (*133*).

These miRNAs that are found to be differentially expressed during various stages of DC development regulate specific signalling pathways. miR-221 was highly upregulated in

immature DCs upon differentiation from human monocytes and is important for their survival was highlighted by increased apoptosis when it was silenced (*134*). Similarly, miR-21 and miR-34a regulate differentiation of human monocyte-derived DCs (MDDC). Target gene analysis revealed miR-21 and miR-34a to regulate expression of the genes WNT1 (coding for Wnt-1) and JAG1 (coding for the Notch ligand Jagged-1) which negatively regulate DC differentiation (*135*).

Some of the most crucial miRNAs that have been identified to regulate different aspects of DC development and functions are as follows.

(a) **miR-155**: It is amongst the most important miRNAs that has been implicated in adaptive immune response. Upregulation of miR 155 is most characteristic feature of both murine as well as human DC maturation. Mice deficient for bic/miRNA-155 were immunodeficient and failed to generate effective $CD4^+$ T cell response (*136*). Another study demonstrated that that miR 155 deficiency led to significant decrease in DC levels of MHC II, CD40, and CD86. This was accompanied by decreased secretion of IL-12p40, IL-12p35, and TNFα, with normal secretion of other cytokines (*137*). Different targets have been identified for miR155. Transcription factor PU.1, which regulates number of myeloid specific genes has been shown to be one such target of miRNA-155 (*138*). Another target of miR-155 is c-Fos and transduction of miR-155–sufficient DCs with c-Fos inhibited their proinflammatory cytokine expression and T cell activation capacity, mirroring the effect of loss of miR-155 (*137*). The authors suggest that c-Fos–containing AP-1 complexes repressed DC maturation, and miR-155 targeting of c-Fos alleviates this and allows maturation to proceed.

(b) **miR-146**: miR-146 is another crucial family of miRNAs that have been shown to regulate DC differentiation. The expression of miR-146a and miR-146b significantly increased monocyte differentiation into iDCs and mDCs. Silencing of miR-146a and/or miR-146b in iDCs and mDCs significantly prevented DC apoptosis and enhanced IL-12p70, IL-6, and TNFα production as well as increase Bcl-2 expression, whereas overexpressing miR-146a and/or miR-146b increased DC apoptosis and reduced cytokine production (*139*).

(c) **Let-7:** It is among the first miRNA identified in *C. elegans*. Role of Let-7i has been conclusively proven in DC maturation. miR-let-7i was upregulated during LPS-induced DC maturation. Downregulation as well as let-7i knockout significantly impede DC maturation. Such let-7i deficient DC, when treated with LPS, are less efficient in stimulating T cell proliferation and instead promoted expansion of the regulatory T cell (Treg) population (*140*).

(d) **miR-142**: miR-142a plays an important role in regulation of antigen processing, presentation and thus T cell activation. Overexpression of miR-142 reduce the phagocytic and antigen processing capacity of DC as well as macrophages (*141*). Along with other pro-inflammatory cytokines like TNFα, DCs also produce IL-6 during maturation. IL-6 is also a key component of LPS-induced endotoxemia and septic shock. IL-6 promoter region has binding site for miR-142 and knock down of miR-142a can inhibit IL-6 production and thus reduce the sepsis induced mortality (*142*). miR-142 is highly expressed in classic FLT3-L–dependent $CD4^+$ DCs, whereas reduced expression has been observed in $CD8\alpha^+$ DC. Moreover, the loss

of miR-142–dependent $CD4^+$ DCs is accompanied by a severe defect in the priming of $CD4^+$ T cells (*143*).

It is pertinent to explore the role of miRNAs in dendritic cells in context of cancer microenvironment. Many techniques were used to do the global miRNA profiling, however with advent of next generation sequencing, the scale, speed and accuracy of sequencing the entire transcriptome has increased tremendously.

1.5.2 Next generation sequencing: The first sequencing method was developed by Sanger and Coulson and it was called plus and minus method. This was the first method to sequence the DNA based on polymerization, not partial hydrolysis. It was successfully used to explore the DNA sequence of bacteriophage ΦX174 (*144*). However, due to its inefficiency, Sanger and colleagues described another method which was known as chain termination or dideoxynucleoside method (*145*). It consisted of a catalysed enzymatic reaction in four separate tubes (each with one type of ddNTP) that polymerizes the DNA fragments complementary to the template DNA of interest. The polymerization was extended until the enzyme incorporated a modified nucleoside which was a dideoxynucleoside triphosphate (ddNTP). The ddNTP terminated the reaction in its site of incorporation. The mixture of different-sized DNA fragments was resolved by electrophoresis on a denaturing polyacrylamide gel, in four parallel lanes. The pattern of bands showed the distribution of the termination in the synthesized strand of DNA. Later, further modification in the ddNTP method were incorporated. Instead of radiolabelled ddNTP, fluorescent molecule tagged ddNTPs were used. However the cost of Sanger method remained very high and the efficiency was

inadequate for large genome sequencing. This has resulted in the reduction of cost of sequencing by more than 10,000 times in the last two decades.

The latest technology in sequencing is using next generation sequencing (NGS), the methodology of which consists of three steps: template preparation, sequencing/imaging and data analysis. NGS from Illumina uses clonal amplification for template preparation. First, the genomic DNA is fragmented randomly into smaller pieces and adapters are ligated onto each end. These pieces are then attached to a solid surface called flow cell. The flow cell is a glass slide with channels and each channel is coated with oligos which are complementary to library adapters. Once the DNA fragments bind to the oligos, then bridge amplification takes place in which single strand DNA attached with a oligo on one end flip and form a bridge through hybridization with nearby complementary primer. Bridge is extended by polymerases to form a double strand bridge followed by denaturation. This cycle keeps on repeating to generate clusters of same fragment at a point. Then sequencing of each cluster takes place through a process called “sequencing by synthesis” in which primers are attached to the forward strands and fluorescently tagged nucleotides are added to the DNA strand. Only one base is added per round. Each of the four nucleotides has an unique label that can be excited to emit a characteristic wavelength. An image is then taken to identify the incorporated nucleotides. The captured image represents the average intensity of colours associated with the ligated nucleotides in each cluster. Each cluster contains about 1000 copies of any template and on a flow cell, there are about 100 million clusters. The latest platform of Illumina sequencer is HiSeq 2500 with Illumina SBS V4 sequencing chemistry. SBS Kit v4 with read length of 2 × 125 bp gives an

output of 900 Gb–1 Tb in a 6-day dual flow cell run. The number of reads passing filter (8 lanes per flow cell) were up to 4 billion single reads or 8 billion paired-end reads.

1.5.3 RNA sequencing: NGS can be used to sequence transcribed RNA templates also through a method known as RNA-Seq. This method can be used for mRNA transcriptome as well as small non coding RNAs like miRNA. Sum total of all the transcribed molecules in a cell is known as transcriptome. Originally it was considered that less than 5% of the genome is transcribed into RNA molecules (*146*). However in 2012, the Encyclopaedia of DNA Elements (ENCODE) consortium reported that 76% of the human genome's noncoding DNA sequences were transcribed (*147*). RNA sequencing has added advantage over genome sequencing. It can be used for detecting alternative splicing sites, transcription start sites or detection of various isoforms (*148*).

One of the most important applications of RNA-seq is sequencing of small RNA molecules. These small RNA molecules like miRNAs are 20-24 nucleotides long and regulate various transcriptional as well as translational events (*149*). As per the miRBase data entry release 21, there are 28645 entries representing hairpin precursor miRNAs, expressing 35828 mature miRNA products, in 223 species (*150*). NGS allows a high throughput categorization of miRNA genes. There are various tools and pipelines that have been developed to give annotation to small RNA after sequencing. These pipelines filter the FastQ format sequencing reads based on the various parameters like size and abundance and then normalization and quantification and expression analysis is done. Further, they are mapped to the reference genome and based on homology and other features, are annotated. Alignment of sequenced reads to reference genome

or transcriptome database is an important step in annotation of small RNA. There are short aligners tools (BFAST, Bowtie etc.) which align continuous reads (not containing gaps result of splicing) to a genome of reference and spliced aligners which align spliced variants to the reference as many reads contains only exon-exon junctions and cannot be aligned directly by short aligners. These splice aligners either can be based on data available in databases about known junctions or it can be de novo splice aligners which allow the detection of new splice junctions and do not need any previous annotation information. Prediction of miRNA is based on the information about hairpin structure as well as conserved region in genome as precursor of miRNAs are in form of hair pin loop structure and their precursors are also mostly phylogenetically conserved. Based on these observations, many algorithms have been developed for ab initio discovery of novel miRNA genes. One such most commonly used algorithm is miRDeep2, which identifies canonical and non-canonical miRNAs based on RNA-seq data. miRDeep2 identified miRNAs with an accuracy of 98.6–99.9% (*148*).

1.6 Dendritic cells in cancer microenvironment:

1.6.1 Anti-cancer role of dendritic cells: Dendritic cells are indispensable components of immunosurveillance system of body. In the initial stage of elimination, stress-associated or damage-associated molecular patterns trigger innate immune activation. Innate recognition of tumor involve dendritic cells through type I interferon signalling which is mapped to antigen-presenting cell compartment (*151*). In Type I IFNR–deficient mice, although the number of pDCs and $CD8\alpha^{+}$ cDCs were normal,

there was a complete absence of accumulation of the $CD8\alpha^+$ DC subset in tumor microenvironment suggesting the IFN mediated role of DC in tumor elimination (*152*). Among different subsets of DC, $CD8\alpha^+$ DCs are particularly effective at capturing antigen from dying tumor cells, and targeting antigen for cross-presentation via the class I MHC processing pathway. This effect is mediated, through the expression of Clec9a (also known as DNGR-1). It has been shown that dying cancer cells exposed a filamentous form of actin which serves as a ligand for Clec9a expressing $CD8\alpha^+$ DCs (*153*).

DC, by virtue of their antigen processing ability and immunomodulatory ability take active participation in elimination as well as equilibrium stage of immunoediting. During elimination stage, dendritic cells are attracted to the transformed cells through chemokine signalling. These chemokines are secreted by first responder macrophages and NKT cells at the site of inflammation. This results in generation of tumor antigens following IFNγ induced death of cancer cells. These are ingested, processed into peptides and presented to T lymphocytes on MHC Class I or Class II molecules by dendritic cells in the draining lymphoid tissues. DC not only directly activate $CD8^+$ T cells against tumor but also further facilitate it by activating tumor-specific $CD4^+$ T cells. If transformed cell survive the elimination stage and immuno-dormant stage accumulating additional mutations, they enter the equilibrium phase. During this, the immune system exerts a selective pressure on developing tumors leading to the elimination of the most sensitive cancer cells but also, as a consequence, to the selection of these resistant tumor cell variants, eventually leading to the escape phase.

Besides the cardinal role as profession antigen presenting cells and co-ordinator of innate and adaptive immune system, many studies have provided evidence that DC can also function as direct cytotoxic effectors against cancer cells. Such DCs are called as killer DC. Different mechanism has been proposed for killer DC mediated killing including Fas-FasL, reactive oxygen species, TNFα and TRAIL (*154*).

1.6.2 Dendritic cells dysfunction in cancer microenvironment:

Progression of cancer is associated with a gradual development of immune suppression. Many factors are involved in causing the failure of immune system and these include defective antigen processing, anergy or inhibition of T cells and activation of different regulatory cells which can actively suppress immune response. Table 1 summarises different immunomodulatory molecules present in cancer microenvironment which affect the DC function. As a critical link between innate and adaptive immune system and key component of anti tumor response, induction of dendritic cells (DC) dysfunction is one of the critical mechanisms employed by the tumor to escape immune surveillance (*18*). Dendritic cells are highly heterogeneous in its function and there are different subtypes originating from the same progenitor that perform different functions at different sites. This heterogeneity of DC phenotype and function is regulated by various factors at different stages of DC differentiation. Therefore, the process of DC differentiation and maturation is highly affected by factors present in DC microenvironment. Tumor cells exploit this vulnerability of DC development and manipulate the DC to either support its progression or become dysfunctional and thereby cause immune suppression. Various mechanisms have been

postulated through which cancer microenvironment manipulate DC depending upon type of cancers and constituents of cancer microenvironment.

Table.1: Soluble factors in cancer microenvironment and their effect on dendritic cells (*155*).

Factors	**Effect on dendritic cells**
IL-10	Inhibit DC differentiation and maturation, Induce apoptosis, generation of regulatory DC.
IL-6	Inhibit the differentiation of $CD34^+$ progenitor cells into DCs and instead trigger their differentiation towards monocytic cells.
GM-CSF	Induce myeloid suppressor phenotype
Transforming growth factor-β (TGF-β)	Inhibit DC maturation, inhibit migration
Chemokine ligand-2 and 5 (CCL2, CXCL5)	Upregulate IL-10 production.
Vascular endothelial growth factor (VEGF)	Impair ability of DC to prime T cells.
ROS, NO	Induce apoptosis, early maturation
Hyaluronic acid	Induce apoptosis
Polyamine	Induce apoptosis
Ganglioside	Inhibit DC differentiation, induce apoptosis
PGE2	Inhibit DC maturation and function

HER-2/Neu	Inhibit affect antigen processing ability
PSA	Inhibit DC differentiation and maturation
Mucin 2 or MUC 2	Inhibit DC maturation and function

1.6.3 Mechanism of DC dysfunction: Various mechanisms have been proposed though which tumor microenvironment affect the DC function. It depends on types of cancer, stage of DC development and other secretory components present in the tumor microenvironment.

(a) **Early maturation and induction of apoptosis**: Many studies have reported an association of cancer progression with low numbers of tumor infiltrating dendritic cells. Though increased number of langerhans cells (LC) were observed in benign skin lesions, it was found to be depleted in invasive melanomas which declined further with metastatic progression (*156*). These results supported the concept that tumor induced apoptosis of either dendritic cells themselves or their precursors. This is relevant, given that tumour cells are known to express or release numerous pro-apoptotic factors such as NO, gangliosides and ceramides that induce DC to undergo apoptosis (*157*). Membrane-associated glycosphingolipids and gangliosides are synthesized by many types of tumor and impair the phenotypic and functional differentiation of DC and also induce apoptosis (*158*). Hyaluronic acid (HA) secreted by gliomas induced apoptosis in dendritic cells through NO induced by $CD44^{-}$HA interactions (*159*). Interaction between Fas/FasL also induced apoptosis in many cancers including endometrioid

adenocarcinoma (*160*). *In vitro* analysis has shown that tumor supernatant directly affected $CD14^+$ cells resulting in up-regulation of nuclear translocation of v-avian reticuloendotheliosis oncogene homologue B (RelB) in cells, resulting in early maturation of dendritic cells as evidenced by high expression of phenotypic maturation markers. These premature DC, though showed characteristics of mature DC, lacked the capacity to produce IL-12 and thus were not capable of allo-stimulation and instead rapidly underwent apoptosis (*161, 162*).

NO, a highly reactive free radical is another important constituent of various cancer microenvironments and is a well-known DNA damaging agent. Elevated expression of NO has been reported in breast, ovarian, gastric, head and neck cancers and can cause apoptosis of DC (*18, 163*). Another small molecule secreted by many types of cancer cells, high mobility groupbox-1 (HMGB1) accelerates cell growth, invasion and angiogenesis and induces apoptosis of macrophages and dendritic cells (*164*). In addition, decreased differentiation of DCs from its precursors, with a significant decrease in the number of circulating DCs have been reported in the peripheral blood of cancer patients suggesting apoptosis in DC precursors. (*165*).

(b) **Altered differentiation and maturation:** Dendritic cell differentiation is a well-studied process. Hematopoietic precursors give rise to progenitor cells that differentiate into immature DCs which migrate to peripheral blood circulation and following antigen encounter, undergo maturation. The soluble factors that affect the DC differentiation process resulting in dysfunctional or pro-tumorigenic DC can be either derived directly from the cancer cells or other associated cells in the

microenvironment (*166*). This is supported by the accumulation of precursors cells of the DC/monocytic lineage at different stages of differentiation in cancer patients (*167, 168*). Presence of such progenitor cells indicate mobilization of precursors from bone marrow or disruption of differentiation from progenitors. Such tumor derived factors include VEGF, IL-6, M-CSF, GMCSF and others. IL-6 and M-CSF secreted from renal carcinoma and pancreatic cancer respectively have been shown to inhibit DC differentiation from $CD34^+$ progenitors and tend to force the lineage towards $CD14^+$ cells. These cells have lower allogenic stimulatory capacity due to decreased expression of MHC II molecules and other co-stimulatory molecules like CD40 and CD80 (*169*). VEGF has also been shown to inhibit DC differentiation from $CD34^+$ progenitor cells in a similar mechanism like M-CSF and IL-6 (*170*). Production of GM-CSF has been reported in several types of human cancers. Although GM-CSF recruit DC and help in differentiation of DC *in vitro*, thereby eliciting anti-tumour immune responses, but chronic production of GM-CSF in cancer site has been found to be associated with accumulation of immature dysfunctional DC (*171*).

Likewise, prostaglandins secreted by stromal cells has also been shown to inhibit differentiation of both mouse and human DC (*172*). Prostaglandins (PGs) are lipids molecules produced enzymatically from 20 carbon fatty acids, particularly arachidonic acid. They are generally produced in most tissues of the body by oxidation of arachidonic acid, although the amount and class of product varies with cell type. In general, three different types of cyclooxygenases (COX) are known to produce prostaglandin. Among the three, COX-1 and COX-3 are constitutively

expressed in most mammalian tissues and regulates normal physiological functions. COX-2 is usually absent from normal tissue and is transiently induced by pro-inflammatory stimuli and tumor promoters to increase the rate of PG formation (*173*). Many studies have shown co-relation between elevated level of PGs with various types of cancers (*174*). One of the most critical products of COX-2 is PGE2 which is pro-tumorigenic in many cancers. Different mechanisms have been proposed for pro-tumorigenic role of PGs including immunosuppression, induction of apoptosis in immune cells, induction of proliferation in cancer cells and enhanced carcinogen metabolism (*175-179*).

(c) **Generation of myeloid derived suppressor cells (MDSC)**: Apart from causing the apoptosis or dysfunction of DC, the cancer microenvironment can also cause the generation of myeloid derived suppressor cells (MDSC) from DC progenitor cells. These MDSC are characterized by hyperactivation of STAT3 (*168*) and expression of CD11b, Gr-1 in mice and expression of CD11b, CD14, CD15, CD33 and MHC class II^{low} in humans (*180*). Different mechanisms have been proposed through which MDSC play pro tumorigenic role and inhibit the antitumor immune response, particularly of T cells. In some cancer, MDSC release nitric oxide. NO suppresses T-cell function through a variety of different mechanisms that involve the inhibition of JAK3 and STAT5 in T cells (*181*), the inhibition of MHC class II expression (*182*) and the induction of T-cell apoptosis. MDSC have also been shown to induce anergic T cells through indoleamine 2, 3 dioxigenase (IDO) secretion and PGE2 (*183*).

(d) **Inhibition of antigen processing ability:** Tumor infiltrating dendritic cells (TIDC) are often found to be inefficient in generating antigen specific T cell response. Antigen processing and presentation ability of TIDC are severely compromised in many types of cancer. Amongst many others, lipid accumulation in dendritic cells due to increased lipid uptake from plasma is one such factor responsible for loss of efficient antigen processing in a cancer microenvironment (*184*).

All these studies demonstrated that tumor microenvironment can make DC dysfunctional or immunosuppressive through various mechanisms. Mitigation of DC dysfunctionality is crucial for an effective anti tumor immune response. There are reports which suggest that many chemotherapy and radiotherapy modalities may help in restoring the DC function. It was shown that cisplatin-treated monocytes gave rise to increased T cell proliferation as cisplatin treatment during DC differentiation up-regulated the IFNβ significantly (*185*). Immunogenicity of cell death that is induced by both chemotherapy as well as radiotherapy is an important aspect of anti tumor immune response and various components of innate immune system including dendritic cells are shown to be important mediators of these response.

1.7 Effect of radiation on differentiation and maturation of dendritic cells: Radiotherapy is one of most widely used therapeutic modality for treatment of cancer. Although radiation therapy is highly focused and directional, many studies have reported significant bystander effect on different immune cells as well as precursors. Because of its apparent ill-effect on immune cells, radiation therapy has traditionally been viewed as immunosuppressive. However, recent studies have suggested more divergent effects of radiation on the immune system, so now it is being re-characterized

as immunomodulatory rather than immunosuppressive (*186*). Radiation produces danger signals and inflammation at the tumor site and these signals may mobilize danger sensing different innate immune cells including DCs. In a recent study, it was shown that efficacy of radiotherapy given as a single, high dose (10 Gy) was dependent on dendritic cells and $CD8^+$ T cells. This high dose of tumor specific radiation activated the tumor-associated dendritic cells which in turn supported the tumor-specific effector $CD8^+$T cells (*187*). This effect of radiation was not limited to a single high dose as another study showed that fractionated radiotherapy given within a time frame of 6 h and a total dose of 7.5 Gy had immunostimulatory effects (*188*). Different mechanisms have been proposed about immunomodulatory role of radiation. In one such study, it was shown that local radiotherapy resulted in the production of IFNβ, which acted on DCs and improved the capacity to cross-present tumor antigens (*189*). Another mechanism proposed involve the release of high mobility group box 1 protein (HMGB1) by dying cancer cells after immunotherapy which further activate DC through TLR4 signalling and promote cross priming $CD8^+$ T cells (*190*). Though these studies demonstrated that radiotherapy could improve the immunogenicity of DCs, the exact underlying mechanism is still not completely elucidated.

1.8 Research hypothesis: As reviewed above, dendritic cells play crucial role in generation of anti tumor response through specialised sensory features and cross presentation ability. Cancer microenvironment directly manipulates DC to be either dysfunctional or pro tumorigenic. As focus is emerging more towards restoration of immune system to fight cancer in form of immunotherapy, understanding of DC dysfunction becomes essential for development of successful immunotherapy. The

hypothesis of this research work is that the tumour microenvironment also affects the distal precursors of dendritic cells thereby affecting DC differentiation resulting in immunosuppressive DC phenotype.

1.9 Objective of present study:

The objectives of present study are:

1) To study the role of cancer microenvironment on differentiation and maturation of DC.
2) To assess the differential miRNA expression in DC affected by cancer microenvironment.
3) To study the effect of progenitor cell irradiation on subsequent differentiation and maturation of DC.

CHAPTER 2

MATERIALS AND METHODS

2.1. Cell culture and *in vitro* assays:

2.1.1 Cell lines:

To study both the *in vitro* and *in vivo* effects of tumor microenvironment on DC differentiation, EL4 lymphoma cells were used for generation of tumor conditioned media as well as development of mice tumor model. EL4 cell line was established from a lymphoma induced in a C57BL/6N mouse by 9,10-dimethyl-1,2-benzanthracene (*191*). EL4 cell line was obtained from the cell repository in the National Centre for Cell Sciences, Pune, India. EL4 cells were maintained in Roswell Park Memorial Institute medium (RPMI-1640) (high glucose- 4mM L-glutamine, 4.5 g/L glucose and 2.0 g/L sodium bicarbonate in 25 mM HEPES buffer with sodium pyruvate) supplemented with 10% fetal bovine serum (FBS), 100 units/ml of penicillin and 100 µg/ml of streptomycin (complete medium). Cells were passaged on alternate days (3 times a week) on attaining confluence. In every passage, about 2 $X10^6$ cells were sub-cultured in 10 ml of complete media and was incubated in a humidified atmosphere at 37^0C in 5% CO_2 or used for further experiments.

A $CD8^+$ T cell hybridoma B3Z was kindly provided by Dr. Satyajit Rath, National Institute of Immunology, New Delhi. The murine T cell hybridoma B3Z (Vα13, Vβ5) expresses an OVA-specific TCR and was first developed by J Karttunen and N Shastri from University of California in 1992 (*192*). The T cell receptor only recognize the 8 amino acid long peptide from ovalbumin (257-264) in the context of $H\text{-}2K^b$ MHC class I molecules. Sequence of the peptide is SER-ILE-ILE-ASN-PHE-GLU-LYS-LEU (SIINFEKL). Additionally B3Z cells have a lacZ operon which is under control of IL-2 promoter. Once

B3Z cells recognize and bind with SIINFEKL peptide with H-2K^b MHC class I, it activates the downstream TCR signaling. Because of IL-2 promoter, lacZ operon gets transcribed and β-galactosidase is synthesized. So B3Z T cell activation can be measured in terms of either β-galactosidase activity using chlorophenol red-β-D-galactopyranoside (CPRG) as substrate or quantification of IL-2 secretion. B3Z cells were also maintained in complete RPMI-1640 media.

2.1.2 Collection of tumor conditioned media (TCM):

EL4 (2X10^6) cells were seeded in 10 ml of complete medium. After 48 hours, when cell density reached ~1X10^6/ml, cells were centrifuged at 2000 rpm for 5 minutes to obtain the supernatant. This culture supernatant was filtered through 0.2 µM syringe filter and used as tumor conditioned media (TCM) for *in vitro* experiments. In some experiments, TCM was also derived from EL4 cells treated with Cox-2 inhibitor NS-398 (10 µM). EL4 cells were seeded along with NS-398 and tumor supernatant media was collected as per the procedure described above.

2.1.3 Generation of bone marrow derived DC (BMDC) and treatments.

BMDC were generated from C57BL/6 mice according to the method of Lutz et al (*193*). Mice were sacrificed and skin and muscles were cut open to expose the femur and tibia bones above and below using dissection scissors. Both the leg bones were then removed by cutting at both the ends leaving as much of the epiphysis intact as possible. Muscles were cleaned off as much as possible using small pointed forceps and scissors. Then femur and tibia were transferred in a dish containing 70% ethanol for surface sterilization for a brief period (1-2 minutes). All the dissection procedures were

carried out in laminar fume hood using only sterile media, instruments, pipette tips and culture dishes. Bones were further transferred into culture media or phosphate buffer saline (PBS) and cleaned properly of skin and muscles. Using forceps and tweezers, bone was cut open at each epiphysis (ends of the bone). It exposed the bone from both the ends. Further bones were held tight with a blunt end forceps and bone marrow cells were flushed out with the help of a sterile syringe (26-28 gauge insulin syringe). Flushing steps were repeated till bone became clear white and translucent. Using the same syringe, bone marrow clumps were disaggregated and single cell suspension was prepared. Cells were transferred into 15 ml falcon tubes. Cells were centrifuged at 1500 RPM for 5 minutes and resuspended in residual media. Cells (5×10^6) were seeded in complete RPMI 1640 medium containing 200 U/ml (20 ng/ml) of murine rGM-CSF and 10 ng/ml of murine rIL-4 in 100 mm non-treated culture plate. On day 3, 10 ml of fresh complete media was added along with rGM-CSF and rIL-4. On day 6, 10 ml spent media was taken out and 10 ml of fresh complete media supplemented with rGMCSF and rIL-4 was added into the culture. On day 8, floating and loosely adherent immature BMDC were harvested, centrifuged and cultured in fresh medium with rGM-CSF (10 ng/ml) for further experimental set up.

In some experiments, DC culture was also supplemented with synthetic PGE2 (10 μM) from day 0 onwards or recombinant IL-10 (50 ng/ml) from day 5 onwards till maturation on day 10.

In some experiments, BMDC culture was supplemented with tumor conditioned media collected from EL4 culture. TCM (20%) was added into the culture on days 0, 3 and 6 along with fresh media with cytokines.

In transwell experiments, bone marrow derived dendritic cells were generated in co-culture with EL4 cells. Bone marrow cells (BMC) (2 X 10^6) were cultured in a 6 well plate with 5 ml complete media containing rGM-CSF (20 ng/ml) and rIL-4 (10 ng/ml). EL4 cells (0.5 X10^6) were cultured in 2 ml complete media using a 0.4 µm transwell (TW) inserts placed in the same well. Every alternate day, EL4 cells were passaged while DC was differentiated as per the standard protocol described above.

2.1.4 Purification of $CD11c^+$ splenic DC and $CD3^+$ T cells:

Purification of $CD11c^+$ and $CD3^+$ cells were performed using magnetic microbeads. In principle, mouse spleen cells are labelled with antibodies against mouse CD11c or $CD3^+$ epitopes which are also tagged with magnetic microbeads. Then the cell suspension is loaded onto a column which is placed in the magnetic field of a MACS^{TM} separator. MS Columns contain a hydrophilic coating which allows rapid filling. This coating is washed out by rinsing the MS Column with buffer before separation. MACS separators are designed in such a way that two strong permanent magnet are vertically placed over a glass platform and column containing microbeads labeled cell suspension is placed in between these two magnets. The ferromagnetic spheres in the column amplify the magnetic field by 10,000-fold, thus inducing a high gradient. Unlabeled cells pass through while magnetically labeled cells are retained within the column. After removal of the column from the magnetic field, the retained fraction can be eluted. The cells labeled with the magnetic beads are retained on the column. Then the column is removed from the magnetic field, to elute the labeled cells retained in the column with the help of a plunger.

Spleens were isolated and single cell suspension was made with the help of a piston and strainer. RBC were lysed using 0.83% ammonium chloride and cells were washed twice with PBS. Cells were resuspended in MACS buffer (PBS with 0.5% bovine serum albumin and 2 mM ethylene diamine tetra acetic acid (EDTA)) and centrifuged at 300 × g for 10 minutes. Supernatant was completely aspirated and cell pellet (10^8) were resuspended in 300 µl of MACS buffer. To this 10 µl of antibody coated magnetic microbeads per 10^7 cells were added. Suspension was mixed and incubated for 30 minutes (2−8°C). Cells were further washed by adding 1 ml of MACS buffer per 10^8 cells at 300×g for 10 min. Supernatant was aspirated completely and cells were resuspended in 500 µL of MACS buffer. MS column was placed onto MACS magnetic separator. The column was washed with 500 µl of MACS buffer and entire 500 µl of cell suspension was loaded on to the column. Flow through containing negatively labelled cells were collected while positively labelled cells were bound to the column due to strong magnetic field. Cells were washed again with 500 µl of MACS buffer. Column was removed from separator and placed in a suitable collection tube and 1 ml of buffer was added to the column. Cells were flushed out forcefully by firmly pushing the plunger into the column. The purity of the cells obtained was assessed by antibody labelling and found to be >90%.

2.1.5 Zbtb46 and Bcl6 knockdown in DC. Bone marrow cells were isolated and cultured for 4 h in serum free media before incubating them with Zbtb46 or Bcl6 siRNA using Xtreme gene siRNA transfection reagent (DNA:reagent::1:5) as per the manufacturer's protocol. After an O/N culture, cells were differentiated with GM-CSF

and IL-4 according to protocol mentioned above. In a different set of experiments, day 8 DC culture was treated with siRNA of Zbtb46 and Bcl6.

2.1.6 Radiation exposure: Bone marrow cells were isolated from mice and irradiated with different doses of radiation (0.5 Gy, 1.0 Gy, 2.0 Gy) using blood irradiator (γ-irradiator, ^{60}Co source). Cells were further harvested and cultured for DC differentiation following standard protocol described above.

2.2 Quantitative and semi-quantitative techniques:

2.2.1 Antigen labelling for flow cytometry

(a) Cell surface labelling: Expression of surface markers (CD40, 80, 86, MHCII (IA/IE), CD19, CD3, CD11c,) was quantified using flow cytometry. Cells (1 x 10^6) were harvested and washed twice with PBS at 2500 rpm for 5 min. Cells were resuspended into minimum volume and incubated with blocking solution for 20 min at RT. Cells were washed and further incubated with respective primary antibodies (0.2 µg/10^6 cells) for 30 min at 4^0C. Cells were washed twice with staining buffer followed by incubation with appropriate secondary antibody (Alexa fluor 488 conjugated anti-rat IgG) for 30 min at 4^0C. Cells were washed twice and finally resuspended into 1 ml PBS. Twenty thousand cells were acquired in Cyflow space^{TM} flow cytometer (Partec, Germany) using Flowmax software analyzed using FCS express software.

(b) Intracellular labeling: For intracellular proteins (CREB, ERK1/2, IL-10, Zbtb46, IL-2) cells were harvested and fixed with 2% formaldehyde for 15 min at 37^0C. Cells were

washed with permeabilization buffer (PBS with 0.5% bovine serum albumin and 0.5% tween 20) at 3000 rpm for 5 min. Cells were resuspended into minimum volume and incubated with the respective primary antibodies at recommended dilutions (CREB: 1:1000, ERK1/2: 1:1000, Zbtb46: 1:250, IL-10 and IL-2: 0.2µg/10^6 cells) for 30 min at 4^0C. Cells were washed in permeabilization buffer and further incubated with secondary antibody for 1 h at 4^0C. Appropriate isotype controls were used. Twenty thousand cells were acquired in Cyflow space^{TM} flow cytometer (Partec, Germany) using Flowmax software analyzed using FCS express software.

2.2.2 Mixed leukocyte reaction

Mixed leukocyte reaction (MLR) is an ex-vivo cellular immune assay that occurs between two allogeneic lymphocyte population (same species but genetically distinct) (*194*). The assay set-up consists of co-culture of purified responder lymphocytes with stimulator cells which could be either an allogenic lymphocyte or leucocytes. MLR could be two way MLR where both the lymphocytes will proliferate against each other's alloantigens or one way where only responder cells proliferate and stimulator cells are prevented from replication by high doses of irradiation or treatment with mitomycin C, a DNA crosslinker to prevent cell replication. Responder T cell proliferates against allogenic major histocompatibility complex (MHC) present on stimulator cells. MLR can be used to assess the capacity of dendritic cells to induce T cell proliferation.

DC (H-2^b) from various treatment groups along with control were exposed to γ-radiation from a ^{60}Co source (25 Gy) and were used as stimulator cells in a co-culture with magnetically purified responder $CD3^+$ T lymphocytes from BALB/c mice in DC:T ratio of

1:4, 1:8, 1:16 and 1:32 in 96 well round bottom plates for 5 days at 37^0C and 5% CO_2. After 5 days, cells were pulsed with ^{3}H-thymidine (1 µCi/well; specific activity 6500 mCi/mmol; Board of Radiation and Isotope Technology, Mumbai, India) overnight. Then cells were harvested on glass fiber filters and the activity was counted in a liquid scintillation counter (Chameleon, Hidex, Germany). The results are expressed as CPM ± s.e.m.

2.2.3 Antigen processing assay

DQ ovalbumin is a self-quenched conjugate of ovalbumin that exhibits bright green fluorescence upon proteolytic degradation. This substrate is labeled with a pH insensitive BODIPY-FL dye and it can be used to study antigen uptake and processing (*195*). Ovalbumin is internalized via the mannose receptor mediated endocytosis pathway and inside the cell, DQ ovalbumin is processed in lysosomal compartment and fluorescence of BODIPY dye is quantified by flow cytometry which represent antigen processing ability of the cells.

DC (5×10^5 cells) and DC (TCM) were incubated with DQ-OVA (1 µg) for 1 h in two different sets at 37^0C as well as at 4^0C. Cells at 4^0C served as control because they were physiologically inactive and could not do phagocytosis and process the DQ ovalbumin. The percentage antigen processing was calculated as the difference between the percentage positive cells at 37^0C and at 4^0C.

2.2.4 Phagocytosis assay: *E.coli* bioparticles are heat or chemically killed *E.coli* bacteria which are covalently linked to fluorescence molecules (FITC). They are used to

study phagocytosis by incubating them with cells at physiological temperature (37^0C) and measuring the fluorescence emitted by the cells using flow cytometry.

DC (1×10^6 cells) and DC (TCM) were incubated with *E.coli*-FITC bioparticles (1:5: cell to bioparticle ratio) for 1 h in two different sets at 37^0C as well as at 4^0C. Cells at 4^0C served as control. The percentage phagocytosis was calculated as the difference between the percentage positive cells at 37^0C and at 4^0C.

2.2.5 Cross presentation and antigen specific T cell proliferation assay:

Cross-presentation is processing and presentation of exogenous antigen to the surface of dendritic cells via MHC I molecules. When an exogenous peptide is supplied to DC, some part of peptide is processed and presented at the surface via MHC I, which in turn activate antigen specific cytotoxic T cells. There are different ways to detect cross presentation capacity of dendritic cells. In this case, DCs were supplied with 8 amino acid long peptide from ovalbumin ($OVA_{257-264}$ or SIINFEKL) and cross presentation was detected at the DC level or by quantifying the activation of SIINFEKL peptide specific activation of cytotoxic T cells.

1. **Detection of cross-presentation by D1.16 antibody:** D1.16 antibody is specific to SIINFEKL peptide only in the context of H-2^b haplotype of MHC I (*196*). Immature DC and DC (TCM) were pulsed with $OVA_{257-264}$ (SIINFEKL) (10 µg/ml) peptide for 4 h and cells were later washed twice with PBS and further cultured in the complete media O/N. Next day, cultured cells were harvested and labelled with anti-mouse PE conjugated D1.16 monoclonal antibody. An isotype control was also included and cells were acquired and analyzed using flow cytometry.

2. **Detection of cross-presentation by B3Z activation**: To evaluate cross-presentation of SIINFEKL peptide by DC and DC (TCM), B3Z T cell hybridoma were used. B3Z is a lacZ-inducible $CD8^+$ T cell hybridoma specific for $OVA_{257–264}$ (SIINFEKL), presented on the murine $H\text{-}2^b$ MHC class I molecule only. The lacZ operon is under control of IL-2 promoter. B3Z T cell activation can be measured in terms of either induced lacZ activity using chlorophenol red-β-D-galactopyranoside (CPRG) as substrate or quantification of IL-2 by activated B3Z cells. Peptide pulsed DC and DC (TCM) were co-incubated with B3Z cells at 1:1 ratio for 24 h at 37^0C in 5% CO_2 atmosphere. Cells were harvested and supernatant was collected to quantify the IL-2. Further, B3Z cells were lysed with CPRG assay buffer. Cell lysate were incubated with CPRG for 18 h at room temperature in dark. Then, absorbance was measured at 570 nm along with a reference wavelength at 650 nm. B3Z cells incubated with un-pulsed DC were taken as control.

2.2.6 Measurement of cytokines:

In different experiments, culture supernatant of DC and other treated groups were tested for the presence of different cytokines (IL-10, IL-12, IL-2) using enzyme linked immuno-sorbent assay (ELISA) kits (*197*). Detection of all three cytokines was carried out using solid phase sandwich ELISA. First the capture antibody against respective cytokine was diluted in suitable buffer in 1:250 dilutions. For IL-10 and IL-2, phosphate buffer was used while IL-12 carbonate buffer was used. Diluted capture antibody (100 µl) was added to 96 well ELISA plates. The plates were sealed using parafilm and kept at 4^0C overnight. Next day, the unbound capture antibodies were removed and wells were washed 3 times with washing buffer. Blocking reagent was added to the wells to

block the free unbound space in the wells and plates were incubated at RT for 1 h. Wells were further washed 3 times using washing reagent by dispensing and aspirating the solution. Appropriate dilution of standards (as per the manufacture's instruction) (Table 2) and samples (100 µl in triplicate) were added to the wells and plates were incubated for 2 h at RT. The wells were again washed 5 times with PBST and thoroughly dried by patting on a blotting sheet. Further biotin tagged detection antibody specific to different epitope of cytokine diluted in the binding buffer was added (100 µl) to the wells and incubated for 1 h at RT. Wells were again washed 5 times using PBST. Streptavidin-HRP conjugate was added to each well and incubated for 1 h at RT. Wells were washed thoroughly and TMB substrate was added to each well and incubated for upto 30 min at RT in dark. After the development of blue color, the reaction was stopped using stop solution. The absorbance of the plate was measured in a plate reader at 450 nm with wave length correction parameter set at 540 nm. Standard curves were generated from absorbance values of cytokine standards. The amount of cytokine in each well was estimated from the equation obtained from standard curve for the cytokines and represented as mean ± s.e.m.

Table 2: **Standard range of different cytokines as per the manufacturer's protocol.**

S.No.	Cytokine	Stand. Range (pg/ml)
1.	IL-10	125-2000
2.	IL-12	62.5-2000
3.	IL-2	6-200
4.	PGE2	7.8-1000

PGE2 detection: PGE2 in culture supernatant as well as in serum samples was detected using competitive ELISA assay. The assay is based on competition between PGE2 present in sample and PGE2-acetylocholineserase conjugates used as a tracer for limited amount of PGE2 monoclonal antibody. Ellman's reagent contains the colorimetric substrate for acetylocholinesterase. There is an inverse relation between signal intensity at 412 nm and amount of PGE2 present in sample as more PGE2 in the sample competitively inhibit the binding of PGE2-acetylocholinesterase conjugate.

Absorbance ∝ [Bound PGE2 Tracer] ∝ 1/[PGE2].

All the dilutions of standards, samples were prepared in ELISA buffer supplied with the kit. Then, 50 µl of each of the standards, samples in duplicate were added to the given wells. Along with these, 100 µl of ELISA buffer was added to wells as non-specific binding (NSB) control and 50 µl of ELISA buffer was added for maximum binding (B0) control. Further 50 µl of prostaglandin E2 AChE tracer (provided in kit) was added to all the wells except blank. Next, 50 µl of prostaglandin E2 monoclonal antibody was added to each well except blank and NSB control. Plate was covered and incubated for 18 h at 4^0C. All the wells were washed with washing buffer provided with the kit by aspirating and dispensing 5 times. Further, 200 µl of Ellman's reagent was added to each well and plate was placed on an orbital shaker in dark for next 60 minutes for the development of color. Plate was removed and absorbance was measured at 405 nm. Absorbance of maximum binding well should be in the range of 0.3 to 1.0 A.U, after subtracting the blank value. Average of NSB value was subtracted from the average of all other values for correcting non-specific absorbance. Standard curve was plotted using %B/B0 vs PGE2 concentration using linear (Y) and log axis (X). B0 is absorbance from maximum

binding well while B is absorbance from other wells. PGE2 concentration in samples was calculated from the standard equation.

2.2.7 Western blotting Western Blotting is an *in vitro* immunoblotting technique to assess the presence of a protein of interest in cell lysate. It was introduced by Towbin, et al. in 1979 (*198*). In this technique, a mixture of proteins is separated based on weight, and thus by type, through polyacrylamide gel electrophoresis. These separated proteins are then transferred from gel to a membrane producing a band for each protein. The membrane is then incubated with primary antibodies specific to the protein of interest. The excess antibody is then washed off and a secondary antibody conjugated with horse redish peroxidase enzyme specific to primary antibody is added. After washing thoroughly, appropriate HRP substrate is added, which gives rise to chemiluminescent signal. This signal is detected either using X-ray photography or CCD camera.

The changes in expression of Zbtb46, phosphorylation in STAT 3/5 in DC, DC (TCM) and DC (Zbtb46 KD) were studied with western blotting. The cells were lysed in 100 µl of 1X gel loading buffer supplemented with 1X phosphatase inhibitor, and 1X protease inhibitor cocktail. The cells were incubated on ice for 15 min and then heated at 95^0C for 5 min. Lysate were further centrifuged at 10,000 rpm for 10 min and stored at -20^0C.

The proteins were separated in 10 % SDS PAGE gel using Tris- glycine electrophoresis buffer. A molecular weight marker was also run in every gel. Gels were run at 90 V for stacking gel and 140 volt for separating gel. The separated proteins from gels were then transferred to polyvinylidene fluoride (PVDF) membrane using semi-wet blotting

technique using transfer buffer and apparatus. To check the efficiency of protein transfer from gel to membrane, proteins were stained with Ponceau S stain. The membrane was blocked in 5% skimmed milk in PBS with 0.05% Tween 20 (PBST) for 1 h at RT and then washed using PBST alone. Membranes were further incubated with primary antibody O/N at 4^0C. Anti Zbtb46 antibody was used in 1:4000 dilution and each of anti-STAT antibodies were used in 1: 2000 dilution. All primary antibodies were diluted in PBST with 4% BSA with 0.01% sodium azide. The membranes were washed thrice with PBST for 15 min each and probed with HRP-conjugated anti-mouse or rabbit IgG antibody (in 5% non-fat milk in PBST). The bands were developed on blot using luminol based chemiluminescent substrate and digitally captured using gel documentation system from Syngene. Protein bands were analyzed and quantified using Image J software.

2.2.8 Assay for apoptosis: Apoptosis can be assessed based on cell cycle analysis using flow cytometry. Cell cycle analysis gives the information about fraction of cells in different phases of the cell cycle. For that, cell are first permeabilised and treated with a fluorescent dye, usually propidium iodide (PI). PI is DNA intercalating dye which stains DNA quantitatively as it binds stoichiometrically to nucleic acids. The fluorescence emission is then proportional to DNA content of a cell. In apoptotic cells, DNA is cleaved into smaller pieces and cells lose significant content of their DNA, so the fluorescence emission (intensity) by apoptotic cells is less than the living cells in G1 phase. Therefore, the presence of cells with DNA content lower than that of G1-cells (sub-G1 peaks) has been considered a marker of cell death by apoptosis. The broad sub-G1

peak by apoptotic cells can be easily distinguished with a narrow sharp peak corresponding to G1 phase of non-apoptotic cells (*199*).

DC treated with TCM at different time points were collected and washed twice with cold PBS and resuspended in minimal residual volume. 1 ml of PI staining buffer was added into each sample, cells were vortexed briefly and after 15 minutes, twenty thousand cells were acquired in a Partec CyFlow® Space flow cytometer using FloMax 2.1™ software and data were analyzed using FCS Express™ software. Cells with less than G1 DNA content were counted as apoptotic cells.

2.2.9 Statistical techniques: Paired t test has been used to calculate statistical significance between two groups in flow cytometry analysis. A 'p value' less than or equal to 0.05 was considered as significant. In *in vivo* tumor experiments, the mean tumor volume between two groups was compared using unpaired t test (unequal variance).

2.3 *In vivo* techniques:

2.3.1 Mice

All mice (C57BL/6 and BALB/c) used in the experiments were bred and maintained in the animal house facility of Bhabha Atomic Research Centre. All experiments were performed as per the guidelines and approval of the Institutional Animal Ethics Committee of Bhabha Atomic Research Centre, Government of India (Project No. BAEC/06/11). Six to eight weeks old C57BL/6 and BALB/c mice were used for the

experiments. The inbred strain of C57BL mice (BL: black) was first created in 1921 by C. C. Little at the Bussey Institute for Research in Applied Biology (*200*). The sub-strain 6 is the most popular of the surviving sub-strains. C57BL/6 mice are black in color and most sensitive to noise and odors. BALB/c is an albino inbred strain of mice. The BALB/c model was first bred by Bagg in 1913 (*201*).

For testing efficacy of COX-2 inhibitor in immunodeficient tumor mice model, SCID (severe combined immunodeficient) mice were used. SCID mice are homozygous for the severe combined immune deficiency spontaneous mutation *Prkdc*^{scid}. PRKDC translate into a DNA dependent protein kinase catalytic subunit which function in DNA non-homologous end joining (NHEJ) required in case of both double-strand break (DSB) repair and V(D)J recombination (*202*). Because of mutation in PRKDC, formation of T cell receptor as well as B cell receptor is hindered and mice become immunodeficient for both T and B cells. SCID mice were purchased from Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Navi Mumbai.

2.3.2 Lymphoma model and treatment:

For testing of drugs under *in vivo* condition, mice lymphoma model was generated, in which mice were injected, sub cutaneous (s.c.) with EL4 lymphoma cells and a solid tumor was allowed to develop. Subcutaneous injections are administered into the sub-cutis, which is the layer of skin directly below the dermis and epidermis, collectively referred to as the cutis. For the injection, $26^{1/2}$ G of needle was used. C57BL/6 mice

were injected with 1 X 10^5 EL4 cells s.c. and randomly segregated into groups as per the experimental requirements.

In one experiment with NS-398, TBM were grouped into: TBM (control), TBM (vehicle) and TBM (NS-398) along with control group without EL4 cell injection. Similarly for combinatorial drug experiments where NS-398 was tested along with CPT, mice were grouped into: TBM (control), TBM (vehicle) and TBM (NS-398), TBM (CPT) and TBM (NS-398 + CPT) with 5 mice in each group.

2.3.3 Tumor volume measurement: In TBM (control), tumor started appearing from day 9 onwards. Tumor volume was measured using digital vernier caliper. For any uneven tumor size, 2 measurements were taken, one with shortest diameter and other with longest diameter. For any even size tumor, two diameters were measured perpendicular to each other. Tumor volume was further calculated by the following formula ($4/3\pi\ (D/2)^3$) (*203*):

Tumor volume (mm^3)= Longest diameter (mm) X shortest $diameter^2$ $(mm)^2$ X 0:53.

2.3.4 Treatment of tumor bearing mice: TBM (NS-398) mice were injected with NS-398 (4 mg/kg of body weight) every day from day 1 till day 12 while TBM (vehicle) were injected with equal volume of DMSO. Injections were given in the intra peritoneal (i.p.) cavity. In those experiments where camptothecin (CPT) was also used either alone or in combination with NS-398, CPT was given i.p. (4 mg/ kg of body weight) on days 3, 6, 9 and 12 to the appropriate groups (Fig 8). Tumor growth was monitored from day 9 after EL4 inoculation.

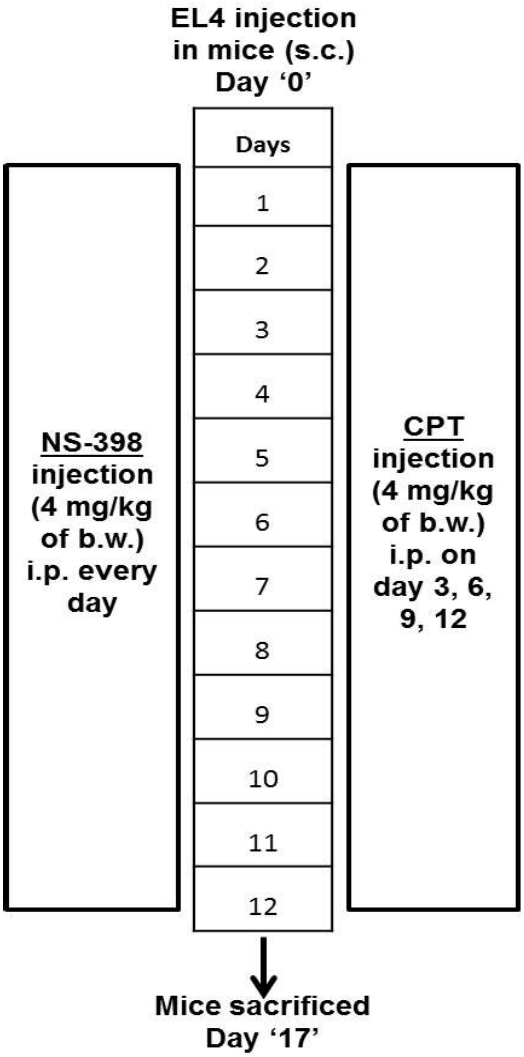


Fig 8: Schematic representation of treatment schedule in mice lymphoma model.

2.3.5 Whole body exposure of mice: For whole body exposure, mice were placed in a covered perspex box with proper ventilation and then exposed to whole body exposure of γ-irradiation (0.5, 1.0, 2.0 Gy) using Bhabhatron II irradiator (BARC, Panacea Biotech Ltd, New Delhi, India) containing a ^{60}Co source at a dose rate of 1 Gy/min.

For adaptive response studies, mice were segregated into four groups (i) control mice (C); (ii) mice exposed to only priming dose (0.1 Gy), (iii) mice exposed to challenging dose only (2 Gy) and (iv) mice exposed to both priming and challenging dose (0.1 + 2 Gy). The time interval between priming and challenging doses was kept at 4 h. Mice were dissected immediately for isolation of bone marrow cells as well as splenic DC as per the experimental requirements.

2.4 RT-PCR analysis, miRNA sequencing and validation.

2.4.1 Estimation of transcription factors by real time PCR:

The expression of Zbtb46, Bcl6, E2-2, ID-2, Batf3 was assessed using real-time PCR. Real time quantitative polymerase chain reaction (QPCR) was developed in1992 (*204*) which allows quantification of DNA, cDNA, or RNA templates as the products accumulate with each cycle of amplification. It is based on the detection of a fluorescent reporter molecule that increases as PCR product accumulate with each cycle of amplification (the level of fluorescence detected is directly proportional to the PCR product yield). The level of fluorescence is continuously monitored through the software and hence the term 'real-time'. Fluorescent reporter molecules include dyes that bind double-stranded DNA (i.e. SYBR® Green I) or sequence–specific probes (i.e. Molecular Beacons®, Amplifluor® probes, Scorpions® probes or TaqMan® Probes). SYBR Green I binds to all double–stranded DNA and is monitored by measuring the increase in fluorescence throughout the cycle.

Total RNA was isolated using RNA isolation kit following the manufacturer's protocol. This RNA isolation procedure is based on solid phase extraction method where cell lysates containing total RNA is passed through a column comprising of silica membrane (*205*). Under an optimal pH and salt concentration of the binding solution, the nucleic acid binds to the silica gel membrane as the lysate solution passes through the column. Guanidinium isothiocyanate (GITC) is used as chaotropic agent to facilitate the binding of nucleic acid and silica membrane. In brief, cells were pelleted down and resuspended in a lysis solution containing β-mercaptoethanol (β-ME) and vortexed for complete lysis of cells. Lysate were directly added into a HiShredder column provided with the kit and placed in a 2 ml uncapped collection tube, and centrifuged for 2 min at full speed (>12,000 rpm). To the homogenized lysate, 1 volume of 70% ethanol was added and mixed well without vortexing. Sample was further loaded on the HiElute Miniprep Spin Column (provided with the kit) and centrifuged for 15 seconds at ≥8000 x g. Flow-through was discarded. RNA samples on the column was first washed with pre-wash solution (700 µl) and then with wash solution (500 µl) provided with the kit for 15 seconds at ≥8000 x g. Column were then transferred to a new 2 ml capped collection tube and 50 µl of RNAse free water was added to the column and centrifuged for 1 min at ≥8000 x g. RNA was eluted in this process. RNA concentration was determined by measuring its absorbance at wavelength of A_{260}/A_{280} nm (Picodrop™ spectrophotometer). A_{260}/A_{280} nm ratio gives an estimate of the purity of the RNA. An optical density ratio of 2.0 (A_{260}/A_{280} nm) indicate a good purity of RNA. Optical density values less than 1.5 indicates ethanol or protein contamination. The RNA sample was then stored at -80ºC.

RNA samples were later reverse transcribed in to cDNA using SuperScript VILO cDNA Synthesis Kit. Reverse transcription leads to synthesis of complementary strand of DNA from RNA. It is followed by polymerase chain reaction (PCR) and the two processes together are known as RT- PCR. One microgram of total RNA was reverse transcribed to cDNA in a reaction containing random hexanucleotide primers, dNTPs and reverse transcriptase using first strand cDNA synthesis kit. The reaction mixture was incubated at 25^0C for 10 min and then 50ºC for 1 h to commence cDNA synthesis followed by incubation at 85ºC for 5 min to inactivate RNase. The cDNA was diluted to 1:10 in PCR water (distilled, de-ionized and UV- treated H_2O) and the samples were stored at -20ºC until required or used for PCR amplification.

Further a PCR reaction was set with the cDNA obtained after reverse transcription. Equal amount of cDNA (0.5 µl) was used for PCR amplification of GAPDH as housekeeping gene and other target genes using 2.5 pmoles of gene specific primers (Table 3). Real-time PCR was carried out in Light Cycler 480 (Roche, Germany). All reactions were performed with SYBR green I in PCR mix and in triplicates. Thermal cycling condition was 10 min at 95^0C, followed by 40 cycles of 2-step PCR consisting of 10 s at 95^0C and 30 s at 55^0C. Ct value was calculated using Light cycler 480 analysis software. The relative expression and significance of different mRNAs in DC (TCM) with respect to DC (control) was calculated using REST^{TM} 2009 V2.0.13 software.

Table 3: List of primers used in the study.

S.No.	Gene name	Primer sequence
1	**Zbtb46**	F: GACACATGCGCTCACATACTG R: TGCACACGTACTTCTTGTCCT
2	**E2-2 (TCF-4)**	F: CGAAAAGTTCCTCCGGGTTTG R: CGTAGCCGGGCTGATTCAT
3	**ATF-like 3 (Batf3)**	F: CAGAGCCCCAAGGACGATG R: GCACAAAGTTCATAGGACACAGC
4	**Bcl6**	F'-CCGGCTCAATAATCTCGTGAA R'-GGTGCATGTAGAGTGGTGAGTGA
5	**ID-2**	F: ATGAAAGCCTTCAGTCCGGTG R: AGCAGACTCATCGGGTCGT

2.4.2 miRNA sequencing

Study of miRNA in dendritic cells in context of tumor microenvironment has been carried out in two parts. First whole transcriptome was sequenced using high throughput next generation sequencing (ILLUMINA) and based on the reads, known and novel miRNA which were differentially regulated were identified. In second step, relevant miRNAs were selected based on sequencing data and available literature and were verified using qRT-PCR. For miRNA study, RNA was prepared from 6 different samples in two biological replicates each. These were:

1) Immature DC (iDC).
2) Immature DC TCM (iDC-TCM).
3) Mature DC (mDC).

4) Mature DC TCM (mDC-TCM).
5) Bone marrow cells from no tumor mice (BMC NT).
6) Bone marrow cells from tumor bearing mice (BMC TBM).

Sample preparation: DC were cultured as per the protocol described above along with tumor conditioned media (TCM). On day 8, iDC and iDC (TCM) were harvested, washed with PBS and stored in RNA later (200 µl) at -20^{0}C. Another set of DC were treated with LPS for 48 h and mature cells were harvested on day 10 and stored in RNA later. Cells stored in RNA later were either sent for RNA-seq or used for validation of the results later using qRT-PCR.

Library preparation and quality control analysis for RNA-Seq:

For RNA-seq analysis, cells were prepared as described above and transported to Nucleome Informatics Pvt Ltd., Hyderabad. RNA isolation, QC check, library preparation, sequencing and bioinformatics analysis was carried out by Nucleome Informatics Pvt Ltd. The methodology carried out is described below.

For RNA sequencing, library preparation was carried out using TruSeq small RNA Library preparation kit. First the 3' and 5' adapters were ligated on the RNA templates on both ends, then RNA was reverse transcribed and amplified to generate cDNA templates. For subsequent cluster generation, cDNA was purified using agarose gel electrophoresis. For quality control analysis cDNA construct were loaded onto Agilent bioanalyser 2100 using high sensitivity DNA chip. The workflow of the sequencing was as follows:

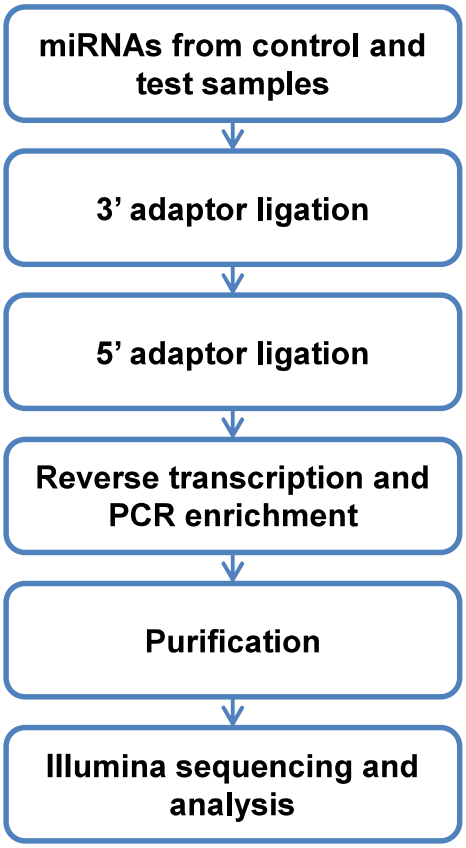


Fig 9: Flow chart of RNA sequencing process.

Sample quality control was carried out by:

(1) Agarose Gel Electrophoresis: To test RNA degradation and potential contamination.

(2) Nanodrop: to test RNA purity (A_{260}/A_{280} nm)

(3) Agilent 2100: To check RNA integrity and quantification.

Small RNA adapter sequences:

RNA 5’ Adapter (RA5), part: 5'-GTTCAGAGTTCTACAGTCCGACGATC-3'.

RNA 3' Adapter (RA3), part: 5'-AGATCGGAAGAGCACACGTCT-3'.

miRNA sequencing and data quality control: cDNA constructs were sequenced using Illumina HiSeq 2500 (4 Dyes based System). Sequencing Chemistry used for the RNA-seq was Illumina HiSeq SBS Kit V4. Fast quality control report was generated based on following criteria:

I. **Raw reads**: Four rows as a unit to calculate the sequence number of each raw data file. It contains 5' primer contaminants, oversized insertion, low quality reads, poly A tags and small tags etc.
II. **Clean reads:** Calculated as Raw Reads. The subsequent analyses are all based on clean reads. Obtained by removing reads with 5' primer contaminants, reads without 3' primer and reads without the insert tag, reads with polyA/T/G/C and by trimming 3'primer sequence.
III. **Raw bases:** (Number of sequences) * (sequence length), use G for unit.
IV. **Clean bases:** (Number of sequences) * (sequence length), use G for unit.
V. **Error rate:** base error rate.
VI. **Q20, Q30:** (Base number of Phil's Read Editor (Phred) value > 20(> 30)) / (Total base number).
VII. **GC content:** (G&C base number) / (Total base number).

miRDeep2 package: The miRDeep2 algorithm was used to identify known and novel miRNAs in high-throughput sequencing data. The package had three modules.

1) **The Mapper module** processes raw sequence output from the Illumina platform and maps the processed reads to the reference genome.

2) **The Quantifier module** sums up read counts for known miRNAs in a sequencing data set.
3) **The miRDeep2 module** identifies known and novel miRNAs in the analyzed high-throughput sequencing data. The input to miRDeep2 is the reference genome, a set of high-throughput sequencing reads and a file with positions of the reads mapped against the genome.

Module gave each miRNA a provisional ID and miRDeep2 score was used to identify the true positive results. miRDeep2 score showed the confidence of being true for the identified miRNA; the higher value, the more confidence of being true.

2.4.3 miRNA validation:

Expression level of miRNA's was measured by the transcript abundance. The higher the abundance, higher was the miRNA expression level. The abundance was counted by counting the reads mapped to the miRNA. Read count was proportional to the miRNA's real expression level as well as to the miRNA length and the sequencing depth. Therefore, the calculated miRNA expression was directly used for comparing the difference of miRNA expression among samples.

miRNA isolation: miRNA was isolated using column based miRNA purification kit following the manufacturer's protocol. In brief, cells were pelleted down at >300 x g for 5 min and washed twice with PBS. Cell pellets were loosened by vortexing and cells were lysed using 500 µl of lysis mix (1:1 ratio of lysis buffer and binding solution). Crude lysates were transferred to a 2 ml centrifuge tube and were centrifuged at 14000 x g for 5-10 min to remove cellular debris. Absolute alcohol was added to clarified lysate in 1:1

ratio and 700 µl of this mixture was added to binding column and centrifuged at 1400 x g for 30 sec. Flow through was discarded and column was washed twice with wash solution provided with kit. Column was centrifuged at same condition in dry state again. Column was transferred to a fresh collection tube and 50 µl of elution buffer was added to the column and centrifuged at 14000 x g for 1 min. miRNA was eluted with the buffer. miRNA concentration (ng/µl) and purity (A_{260}/A_{280} nm) was confirmed in NanoDrop™ 2000 spectrophotometer.

First stand cDNA synthesis: miRNA concentration of the purified samples was adjusted to 5 ng/µl using nuclease free water. First stand cDNA was prepared following manufacturer's protocol. In brief, miRNA reaction mix per well was prepared in following ratio:

Table 4: Preparation of first strand cDNA synthesis reaction mixture.

S.No.	Component	Volume
1	5 X reaction buffer	2 µl
2	H_2O	5 µl
3	Enzyme mix	1 µl
4	RNA template	2 µl
	Total	**10 µl**

A master mix was prepared without miRNA template, mixed properly and later 2 µl of miRNA template were added to each master mix respectively.

cDNA master mix with respective miRNA templates were incubated at 42^0C for 60 min, and later at 95^0C for 5 min. After the reaction, tubes were immediately transferred to ice or 4^0C to cool for 30 min and further shifted to -20^0C for long term storage.

Validation of miRNA by qRT-PCR: Each miRNA primer was first resuspended in 220 µl of RNAse free water, mixed and 50 µl aliquots were prepared. Also, all cDNA templates prepared were diluted to 1:80 dilution using nuclease free water. RT-PCR reaction was set up as per the manufacturer's protocol. In brief, a reaction master mix was prepared with following combination:

Reaction mix (2X): 5 µl/ well.

Primer: 1 µl/ well.

Master mix was properly mixed and aliquoted into respective well of 96 well white plate. Further, 4 µl of diluted cDNA was added to the respective well and plates were sealed and centrifuged at 1500 x g for 1 min. The reaction was carried out in a LC480 light cycler under the following conditions.

Table 5: RT-PCR setup for miRNA quantification.

S. No.	Reaction	Conditions
1	**Activation:**	95^0C, 10 min
2	**Extension:** **(X 45 cycle)**	95^0C, 10 Sec. 60^0C, 1 min. (ramp rate: 1.6^0C/S)

3	**Melting**	95^0C, 5 sec. 65^0C, 1 min. 95^0C, continuous.
4	**Cooling:**	40^0C, indefinitely

The qPCR assay was also followed by a melt curve analysis to confirm specificity of the amplified product and exclusion of false positives.

Table 6: List of miRNA target seqences used in RT-PCR experiments.

S.No.	miRNA primer	Target sequence
1	mmu-miR-155-3p	5'CUCCUACCUGUUAGCAUUAAC
2	mmu-miR-155-5p	5'UUAAUGCUAAUUGUGAUAGGGGU
3	mmu-mir-146a-3p	5'CCUGUGAAAUUCAGUUCUUCAG
4	mmu-miR-365-2-5p	5'AGGGACUUUCAGGGGCAGCUGUG
5	mmu-miR-187-3p	5'UCGUGUCUUGUGUUGCAGCCGG
6	mmu-miR-142-3p	5'UGUAGUGUUUCCUACUUUAUGGA
7	mmu-miR-7676	5'AGGGCAGUAUGAUGGCCUCUGAU

CHAPTER 3:

RESULTS

Tumor progression is associated with dysfunction of immune system (*32*). Dendritic cells, being one of the key components of anti tumor immunity, are highly vulnerable to different components of cancer microenvironment. Many studies have shown that tumor infiltrating dendritic cells undergo apoptosis or their maturation is manipulated to hinder effective anti-tumor immune response (*160, 162*). However, how cancer microenvironment affects the differentiation of dendritic cells from early progenitors remains an open question and largely unexplored. Many recent findings have deciphered the transcriptional network of dendritic cells that determine lineage and thus function of dendritic cells (*206*). In this study, we have explored the effect of cancer microenvironment on lineage specific transcription factors. miRNA play an important role in regulation of transcription factors and in this context, we have also carried out a global miRNA profiling of dendritic cells and their progenitors in presence or absence of cancer microenvironment. Effect of radiation on bone marrow progenitor cells and subsequently on dendritic cells in their normal course of differentiation or in presence of tumor conditioned medium has also been studied.

The results chapter is subdivided into 3 parts. 3.1 describes the *in vitro* and *in vivo* effects of cancer microenvironment on differentiation and maturation of dendritic cells. Though IL-10 was identified to be secreted by DC in presence of TCM from day 5 onwards through ERK-CREB mediated signalling, it was not the primary soluble factor responsible for TCM mediated DC dysfunction. From the several lineage specific transcription factors which are responsible for lineage decisions of dendritic cells, Zbtb46 was identified as an important target and mediator of cancer induced DC dysfunction. The primary soluble mediator which affected IL-10 and Zbtb46 was

identified as PGE2 secreted by tumor cells and inhibition of Cox-2 in EL4 cells by the compound NS-398 was able to restore DC function both *in vitro* and *in vivo* and significantly reduced tumor burden in C57BL/6 lymphoma model.. Chapter 3.2 describes the differentially expressed miRNA in immature and mature dendritic cells treated with tumor conditioned medium as well as in bone marrow cells of normal and tumor bearing mice and Chapter 3.3 describes the increase in immunogenicity of bone marrow derived dendritic cells (BMDC) following irradiation of progenitors cells. This increased immunogenicity was able to alleviate the tumor induced suppression of DC differentiation and maturation.

3.1 Effect of tumor microenvironment on differentiation and maturation of DC.

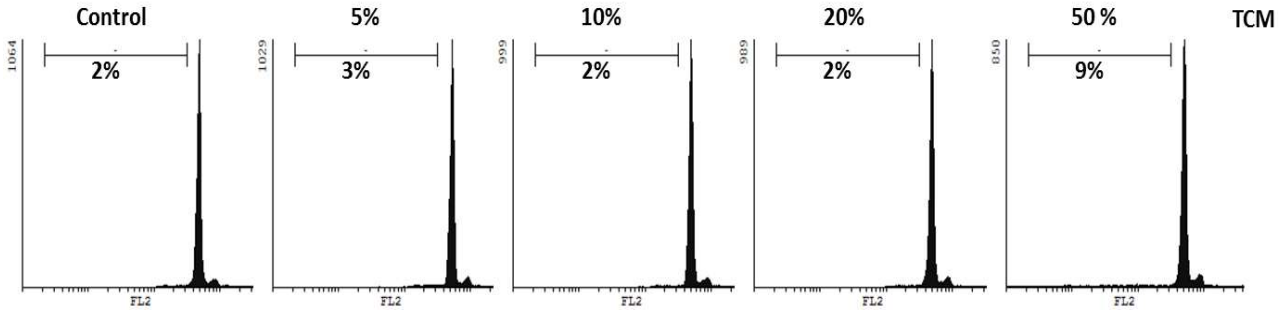
In order to study the role of cancer microenvironment on dendritic cells under *in vitro* conditions, BMDC from C57BL/6 mice were treated with tumor conditioned medium (TCM) either during differentiation, maturation or both. To study *in vivo* effect of tumor microenvironment, $CD11c^+$ splenic dendritic cells from tumor bearing mice were used.

3.1.1. Effect of cancer microenvironment on DC differentiation *in vitro* and *in vivo*:

3.1.1 (a) TCM derived from EL4 lymphoma did not have cytotoxic effect on DC.

As TCM from EL4 cells were used to study the effect of cancer on DC differentiation, the cytotoxic effects of EL4 derived TCM was assessed on DC progenitor bone marrow cells (BMC). BMC cultured with different concentrations of TCM (5%, 10%, 20%, 50%) for 24 h did not demonstrate any cytotoxicity (Fig 10a). For further experiments, 20% TCM concentration was chosen. DC were differentiated in 20% TCM and cytotoxicity of TCM was evaluated on days 3, 5 and 7. No difference in cell death was observed between untreated and TCM treated DC (Fig 10b).

(a)



(b)

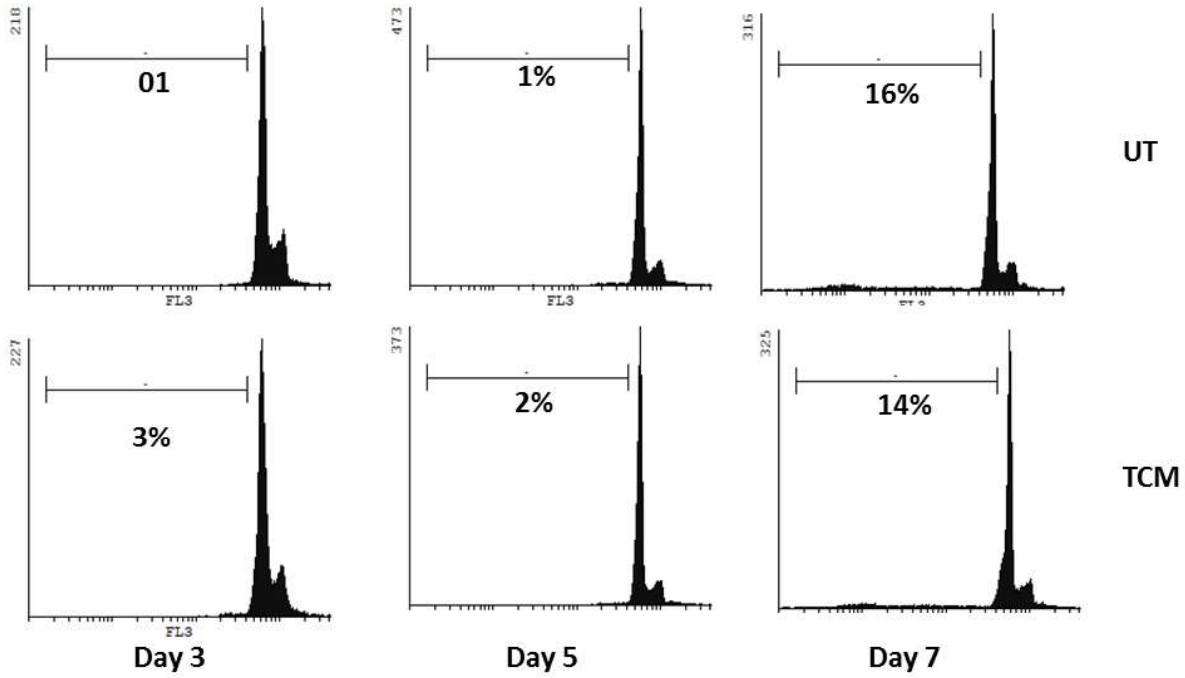


Figure 10- Effect of tumor conditioned media on BMC viability: Bone marrow cells were isolated from C57BL/6 mice and cultured with (a) different concentration of TCM (5- 50%) along with control for 24 h. (b) TCM (20%) for days 3- 7. Cells were labelled with PI staining solution and twenty thousand cells were acquired in Partec Cyflowspace^{TM} flow cytometer and cells having sub G1 DNA content were enumerated as apoptotic cells.

3.1.1 (b) Tumor conditioned media suppressed differentiation of DC *in vitro*: The effect of tumor microenvironment on phenotypic maturation of DC was studied using two different systems under *in vitro* condition.

1. (a) Treatment with TCM during DC differentiation: Dendritic cells were differentiated in presence of TCM (20%) from day 0 till day 8.

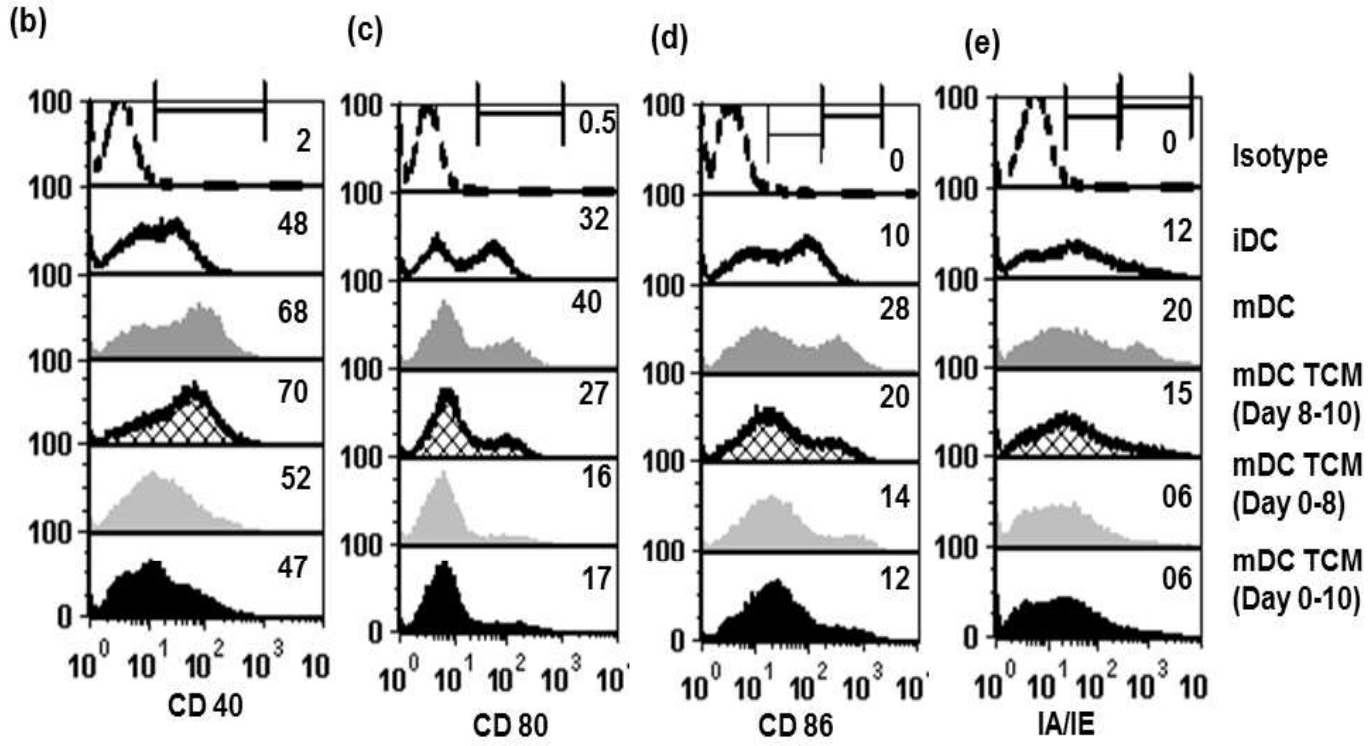
(b) Treatment with TCM during DC maturation: Control DC differentiated without TCM and underwent maturation in presence of TCM (20%) from 8 to day 10.

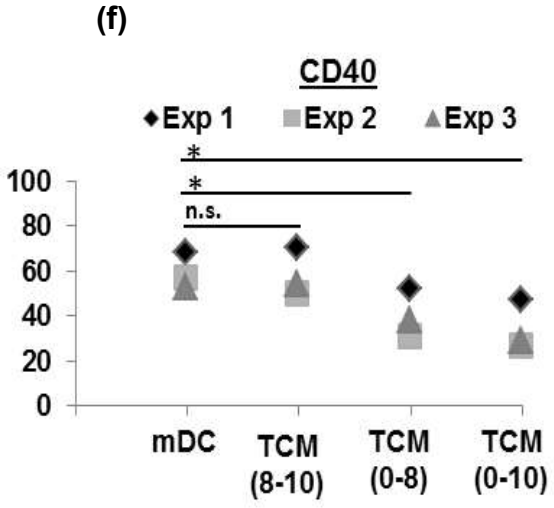
(c) Treatment with TCM during differentiation and maturation: DC underwent differentiation and maturation in presence of TCM from day 0 to day 10. (Fig 11a)

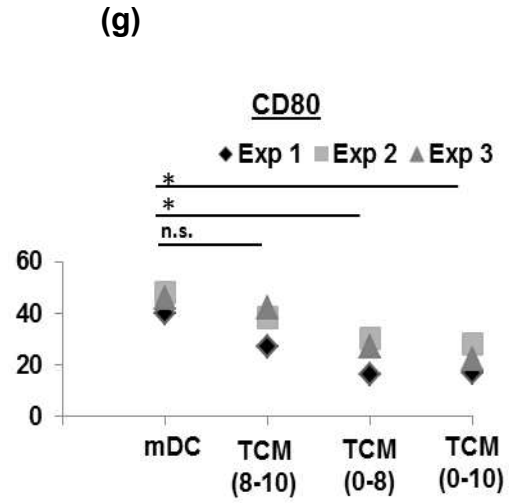
2. DC were differentiated from day 0 to day 10 in a co-culture with EL4 cells through a transwell insert.

Following these treatments, expression of phenotypic maturation markers CD40, CD80, CD86 and IA/IE (MHC II) were analysed. The LPS induced DC maturation process resulted in increase in expression of all the maturation markers in mDC as compared to iDC. Expression of CD40 increased from 48% in iDC to 68% in mDC and it was unchanged (70%) in mDC TCM (Day 8-10). However in mDC TCM (Day 0-8) expression of CD40 decreased to 52% and in mDC TCM (Day 0-10) expression was further reduced to 47% (Fig 11b). Expression of CD80 decreased from 40% in mDC to 27% in mDC TCM (Day 8-10) and further reduced to 16% and 17% in mDC TCM (Day 0-8) and mDC TCM (Day 0-10) respectively (Fig 11c). Similar trend was also observed in CD86 and MHCII expression. For $CD86^{high}$, the percentage positive cells were 28%, 20%, 14% and 12% in mDC, mDC TCM (Day 8-10), mDC TCM (Day 0-8) and mDC TCM (Day 0-10) respectively (Fig 11d). In case of MHC II^{high}, the percentage positive cells were 20%, 15%, 6% and 6% in mDC, mDC TCM (Day 8-10), mDC TCM (Day 0-8) and mDC TCM (Day 0-10) respectively (Fig 11e). Percentage positive value of CD40, 80, 86^{high} and IA/IE^{high} ($MHCII^{high}$) in three independent experiments is shown as histogram in fig 11f, g, h, i respectively.


(a)
TCM
Bone marrow cells
GMCSF & IL4
Day 0- 8
(Differentiation)
Immature BMDC
TCM
LPS
Day 8- 10
(Maturation)
Mature BMDC


(b)
(c)
(d)
(e)
CD 40
CD 80
CD 86
IA/IE
Isotype
iDC
mDC
mDC TCM (Day 8-10)
mDC TCM (Day 0-8)
mDC TCM (Day 0-10)


(f)
CD40
Exp 1
Exp 2
Exp 3
n.s.
mDC
TCM (8-10)
TCM (0-8)
TCM (0-10)


(g)
CD80
Exp 1
Exp 2
Exp 3
n.s.
mDC
TCM (8-10)
TCM (0-8)
TCM (0-10)

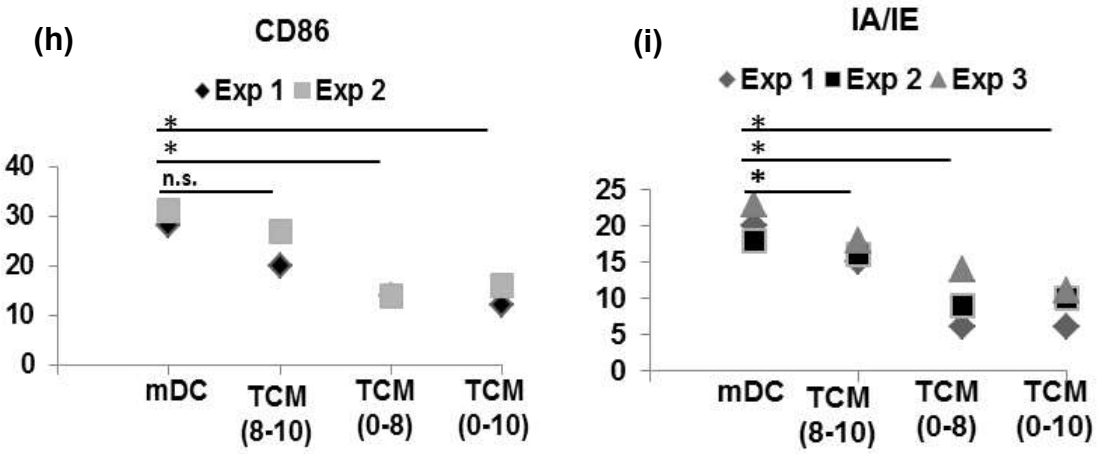
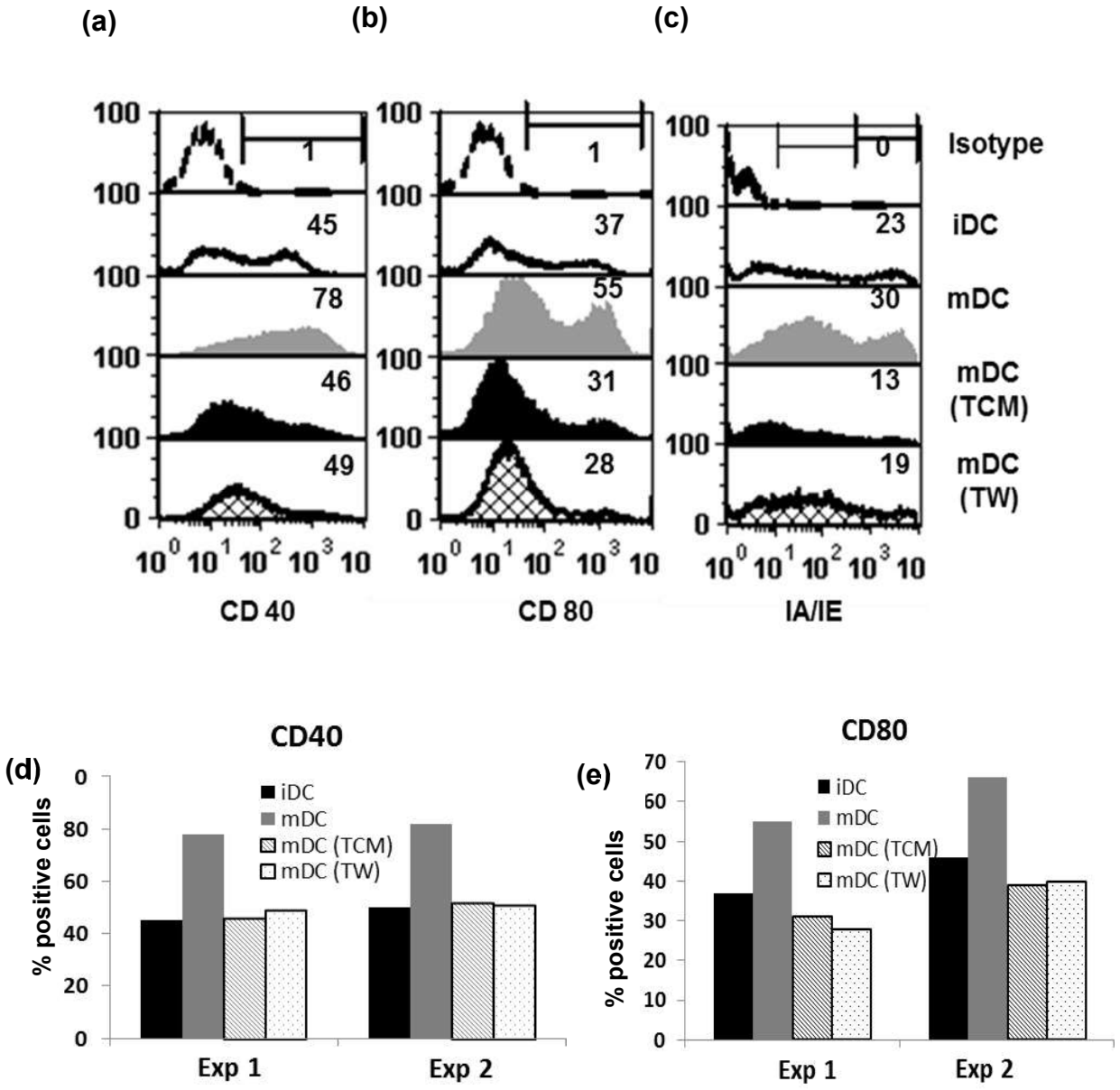


Fig 11. TCM suppressed the differentiation of DC (*in vitro*): (a) Schematic representation of TCM treatment schedule of dendritic cells culture. Bone marrow cells were cultured in DC differentiation media and DC culture was supplemented with 20% TCM during differentiation period only (day 0-8), during maturation period (day 8-10) only and throughout differentiation and maturation period (day 0-10) along with control DC without TCM. Expression of (b) CD40, (c) CD80, (d) CD86, and (e) MHCII (IA/IE) were analyzed in all the groups by flow cytometry. Numbers in histogram represent the percentage of positive cells. Three independent experiments were carried out and respective histograms of the markers in each experiment are presented together in fig 11 f-i (*$p<0.05$).

3.1.1 (c) EL-4 co-culture suppressed differentiation of DC *in vitro*:

In TW experiments where DC were co-cultured with EL4 cells, expression of CD40, 80 and MHC II were 78%, 55% and 30% respectively in mDC and it was downregulated to 49%, 28% and 19% respectively in case of mDC (TW) (Fig 12a-c). This decrease was comparable to effects of TCM on BMDC and demonstrates that TCM derived soluble

mediators downregulated phenotypic maturation of BMDC *in vitro* during differentiation. Two independent transwell experiments were carried out and results of both the experiments are shown as histogram in fig 12d-f.



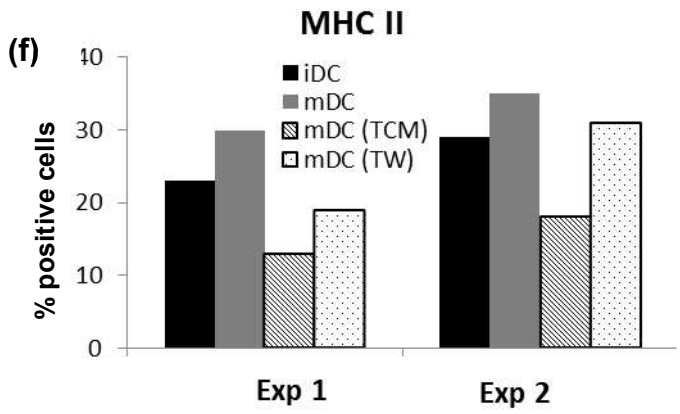


Fig 12: Effect of co-culture of EL4 cells on DC differentiation: DC were differentiated in 6 well plate and EL4 cells were co-cultured using a 0.22 µm transwell insert. DC with and without TCM were taken as control. Expression of (a) CD40, (b) CD80, (c) MHCII was analyzed in all the groups by flow cytometry. Numbers in histogram represent the percentage of positive cells. Expression of (d) CD40, (e) CD80 and (f) MHCII in two independent experiments are plotted as histogram.

3.1.1 (d) Effect of cancer microenvironment on splenic dendritic cells (*in vivo*):

Dendritic cells generated in cancer microenvironment *in vitro* showed downregulation of maturation markers. In order to understand its effect under *in vivo* conditions, maturation status of $CD11c^+$ splenic DC isolated from tumor bearing mice were compared with $CD11c^+$ splenic DC from control mice. Expression of CD40, CD80 and MHCII were downregulated in DC (TBM) from 65% to 38%, 45% to 20% and 45% to 24% and 46% to 26% respectively when compared to control (Fig 13a-d). Experiments were carried out in triplicates and data from all three experiments are summarized in fig 13 (e).

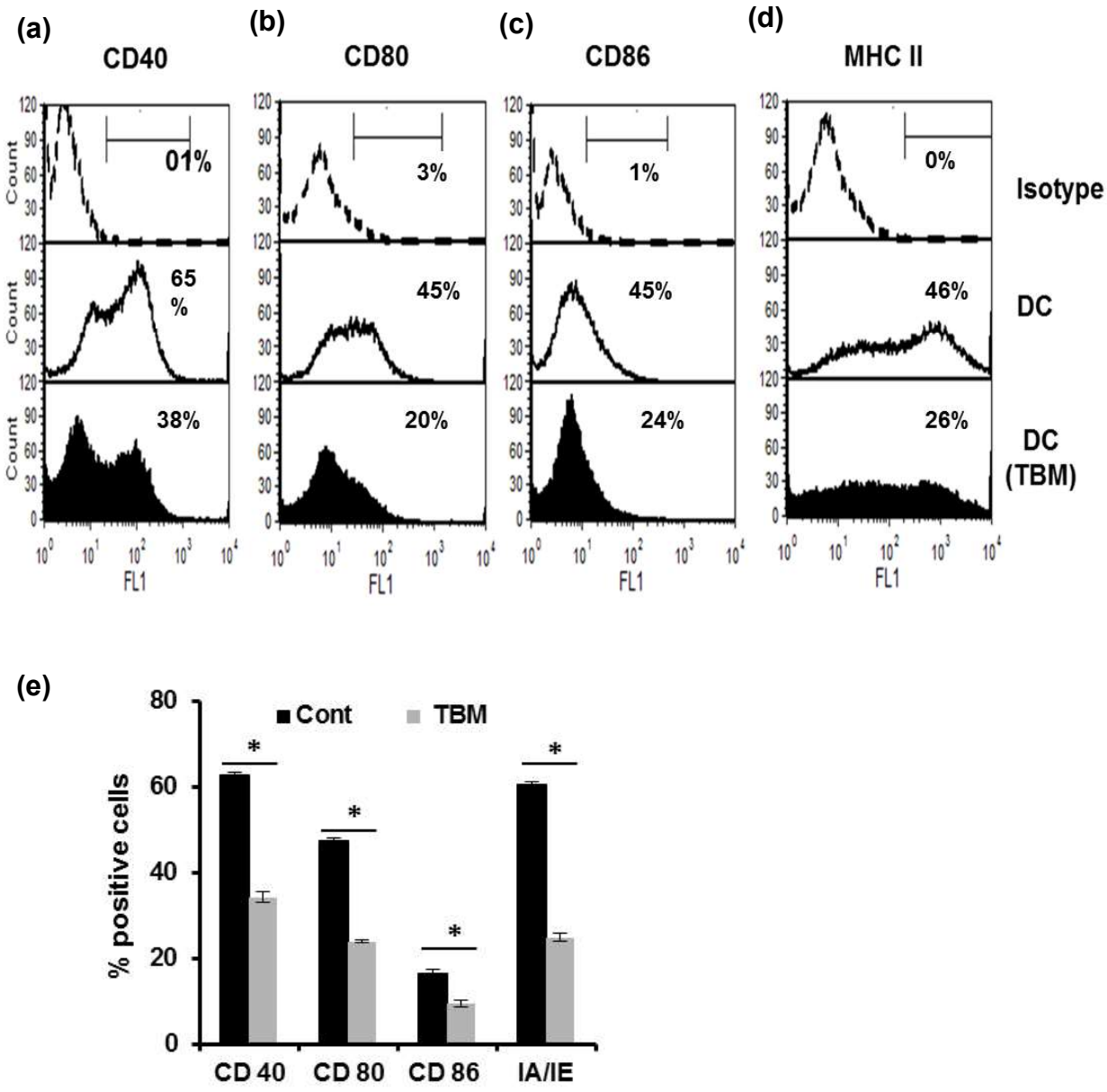


Fig 13: Effect of tumor microenvironment on *in vivo* generated splenic dendritic cells (sDC). sDC were isolated from C57BL/6 mice using CD11c magnetic microbeads and cells were analyzed for the expression for (a) CD40, (b) CD80 and (c) CD86 (d) MHC II using flow cytometry. (e) Experiments were carried out in triplicates and mean expression of all three markers were plotted in figure 13e as mean±s.e.m. (*$p<0.05$)

3.1.1.e Effect of cancer microenvironment on functional status of DC:

Both *in vitro* and *in vivo* generated DC showed marked downregulation of phenotypic maturation markers in presence of cancer microenvironment. In order to understand if down regulation of phenotypic maturation markers also affected functional properties of DC (TCM), they were tested for their functional competency through assays for antigen processing and T cell activation capacity.

(i) TCM downregulated antigen processing capacity of DC: Antigen processing ability was evaluated using DQ-ovalbumin dye. Difference between percentage positive cells at 37^0C and 4^0C were taken as percentage antigen processing. Antigen processing decreased from 36% in control DC to 29% in case of DC TCM (8-10) while it decreased to 8% in case of DC TCM (0-8) and to 10% in case of DC TCM (0-10) (Fig 14).

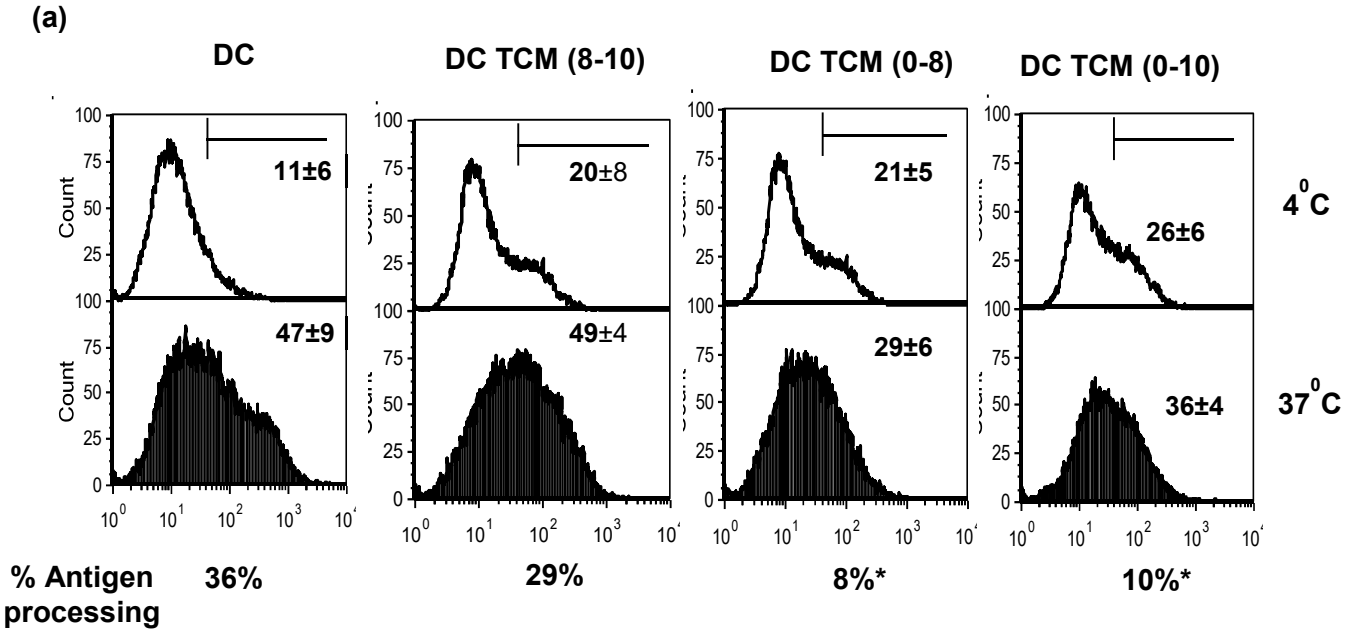


Fig 14: TCM decreased antigen processing ability of DC. (a) Antigen processing ability was quantified as the difference between the percentage of DQ-OVA positive

cells at 37^0C and at 4^0C in DC, DC TCM (8-10), DC TCM (0-8), DC TCM (0-10). Experiment shown here is a representative of 3 independent experiments. Value in histogram is mean ± s.e.m. % antigen processing is calculated based on one experiment (DC vs DC TCM (0-8) and DC vs DC TCM (0-10) *p<0.05).

(ii) TCM downregulated allogenic T cell activation capacity of DC: Dendritic cells express high levels of MHC molecules on their surface due to which they can activate allogenic T cells. DC and DC (TCM) generated from C57BL/6 (H-2^b) mice were incubated with allogenic T cells from BALB/c mice (H-2^d) and T cell proliferation was measured using ^{3}H-thymidine incorporation. Fig 15a shows that the proliferation of T cells against allogenic antigen was also significantly down regulated when co-cultured with DC (TCM) as compared to control DC in all the DC:T cell ratios (1:4, 1:8, 1:16, 1:32) (p<0.05).

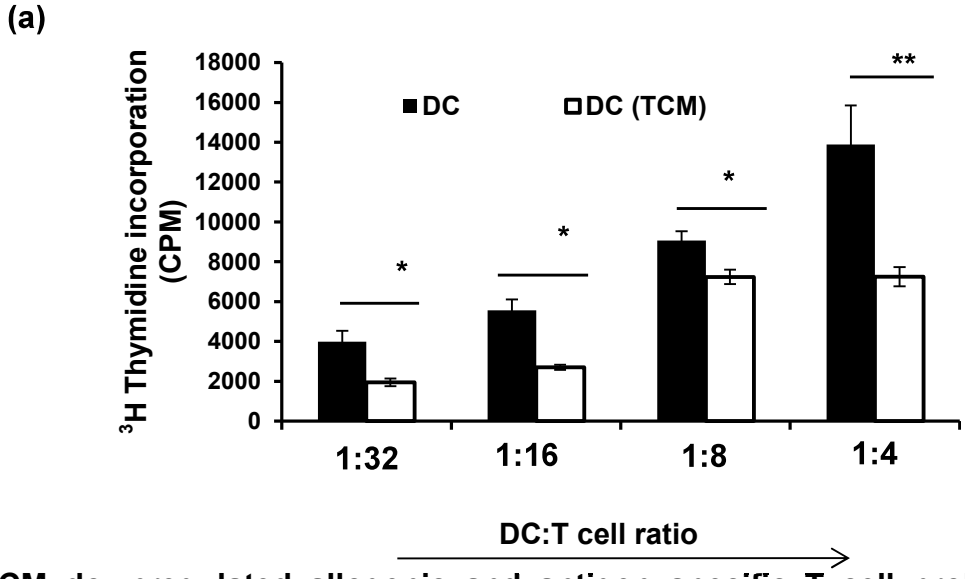


Fig 15: TCM downregulated allogenic and antigen specific T cell proliferation capacity of DC: (a) Allogenic T cell proliferation capacity was assessed by MLR. Irradiated stimulator DC (H-2^b) and DC (TCM) were co-cultured with allogeneic T cells

(H-2^d) from BALB/c mice in different DC to T cells ratios (1:4, 1:8, 1:16, 1:32) for 5 days. T cell proliferation was measured by ^{3}H thymidine incorporation. Values are mean ± s.e.m. of six replicates. *$p < 0.05$, **$p < 0.01$.

(iii) TCM downregulated cross presentation ability of DC: Dendritic cells play very crucial role in anti tumor immune response mainly due to their ability to cross present exogenous antigens from tumor cells to $CD8^+$ T cells via a process called cross presentation. As the antigen processing ability of DC was downregulated in presence of TCM, we next assessed cross presentation capacity of DC (TCM).

SIINFEKL peptide pulsed DC and DC (TCM) were analysed for the presentation of peptide in the context of MHC I (H-2^b) using D1.16 antibody. Flow cytometric analysis showed a 2 fold decrease in cross presentation (D1.16 labelling) in DC (TCM) in comparison to DC control (Fig 15b). Further, these peptide pulsed DC were incubated with B3Z cells ($CD8^+$ T cell hybridoma expressing β-galactosidase under IL-2 promoter) and activation of B3Z cells were analysed. DC (TCM) showed downregulation of intracellular IL-2 from 36% to 13% in comparison to DC control (Fig 15c). Activation of B3Z cells was also measured in terms of β-galactosidase activity. iDC (TCM) showed more than 2 fold decrease in activity than iDC control (Fig 15d).

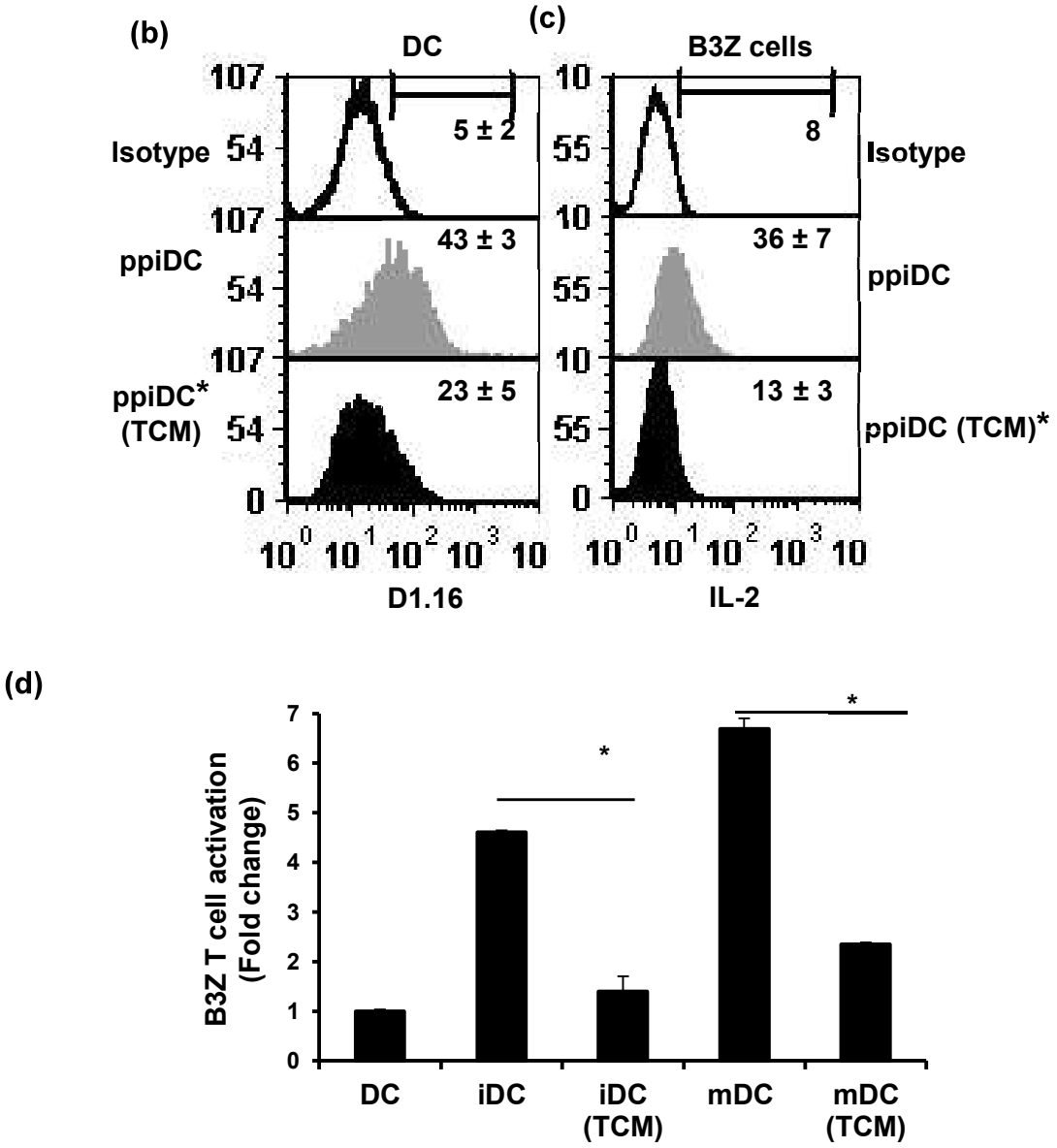


Fig 15: TCM downregulated allogenic and antigen specific T cell proliferation capacity of DC (TCM): (b) SIINFEKL peptide pulsed DC and DC (TCM) were evaluated by D1.16 labelling using flow cytometry. (c) Intra-cellular IL-2 labelling in B3Z cells co-cultured with SIINFEKL peptide pulsed DC and DC (TCM). The numbers in histograms are mean ± s.e.m. of percentage positive cells from triplicates. (d) Activation of B3Z T cells was evaluated by quantifying the expression of galactosidase in B3Z cells co-cultured with SIINFEKL peptide pulsed DC and DC (TCM) by using CPRG as substrate.

CPRG absorbance was taken at 570 nm. Data are mean ± s.e.m. of triplicates (*p<0.05).

(iv) TCM induced suppression of IL-12 secretion:

IL-12 secreted by DC plays a critical role in development of immunocompetent DC and is very important for generating a T_H1 response. On day 8, IL-12 was quantified in DC culture and it was observed that TCM down regulated the secretion of IL-12 in iDC (TCM) by several fold in comparison to iDC (Fig 16).

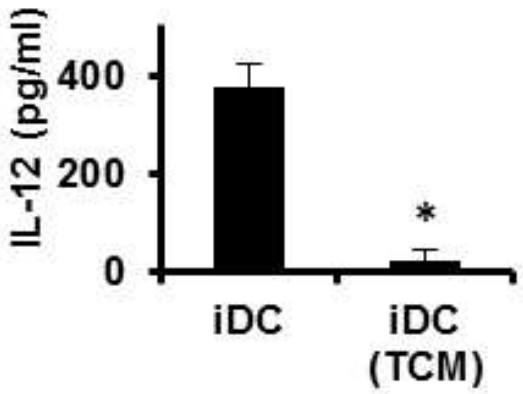


Fig 16: TCM downregulated IL-12 secretion: Culture supernatant was collected on day 8 of DC culture. Secretion of IL-12 was quantified using ELISA. Data are mean ± s.e.m. of triplicates (*p<0.05).

3.1.2 Role of immunosuppressive IL-10 in TCM induced DC dysfunction:

3.1.2 (a) TCM induced IL-10 secretion by dendritic cells: TCM showed downregulation of phenotypic and functional maturation of dendritic cells. Earlier studies have also shown the presence of anti-inflammatory, immunosuppressive IL-10 cytokine in cancer microenvironment secreted by either tumor cells itself or by infiltrating immune cells (*207*). It was pertinent to explore the effect of TCM in context of IL-10 by DC.

We did not observe any detectable levels of IL-10 in EL4 derived TCM. Then DC (TCM) culture was evaluated for the presence of IL-10. Kinetics of IL-10 secretion was assessed in TCM treated DC culture. Control DC did not secrete significant level of IL-10 through the course of differentiation. However DC (TCM) commenced IL-10 secretion from day 6 onwards during differentiation (Fig 17a). On day 8, there was 5-10 fold increase in IL-10 of iDC (TCM) as compared to control iDC. In mature DC, secretion of IL-10 increased upto 1200 pg/ml and there was no significant difference in IL-10 secretion by mDC and mDC (TCM) (Fig 17b).

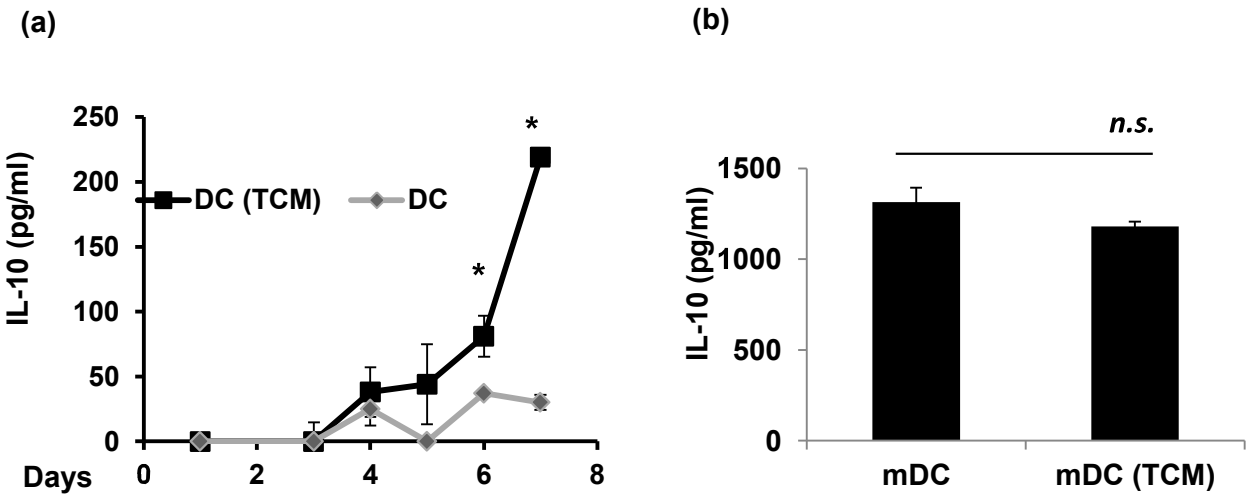


Fig 17: TCM induced secretion of IL-10 in DC: DC and DC (TCM) culture supernatants were collected from day 1 to day 10 and IL-10 was quantified using ELISA. (a) Kinetics of TCM induced IL-10 secretion (days 0–7) monitored by ELISA. (b) IL-10 secretion during maturation (day 10 supernatant) (*$p<0.05$, n.s. not significant).

3.1.2 (b) TCM activated ERK-CREB pathway to induce IL-10 secretion: The transcription factor cAMP response element binding protein (CREB) plays an important role in induction of IL-10 (*208*). As the TCM induced IL-10 secretion in iDC, the

activation of CREB in DC (TCM) and BMDC from TBM were analysed. Western blot (Fig 18a) as well as flow cytometry analysis (Fig 18b) showed upregulation of phopho-CREB in both DC(TCM) and DC (TBM).

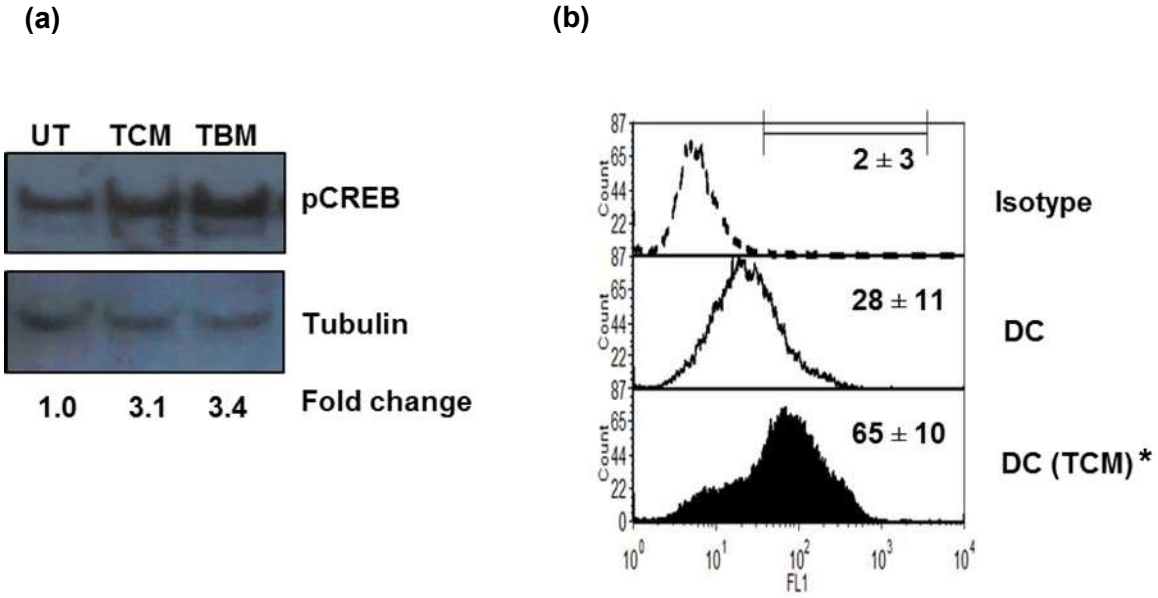


Fig 18: **TCM induced IL-10 through ERK/CREB signalling axis:** (a) DC (TCM) and DC (TBM) were analysed for the basal level of phospho-CREB though western blot analysis (b) Flow cytometry analysis of phospho-CREB expression. Twenty thousand cells were acquired. Number in histogram represents percentage positive cells in triplicates (mean ± s.e.m.) (*$p<0.05$).

Phosphorylation of CREB is known to be regulated through upstream ERK signalling. So DC (TCM) and DC were treated with ERK1/2 inhibitor PD98059 and CREB phosphorylation as well as IL-10 expression was analysed using flow cytometry. TCM induced ERK and CREB phosphorylation in DC (TCM) compared to DC. However when PD98059 was added, it downregulated the TCM induced ERK activation in DC (TCM)

and subsequently CREB phosphorylation as well as IL-10 expression was also reduced (Fig. 18 c-e).

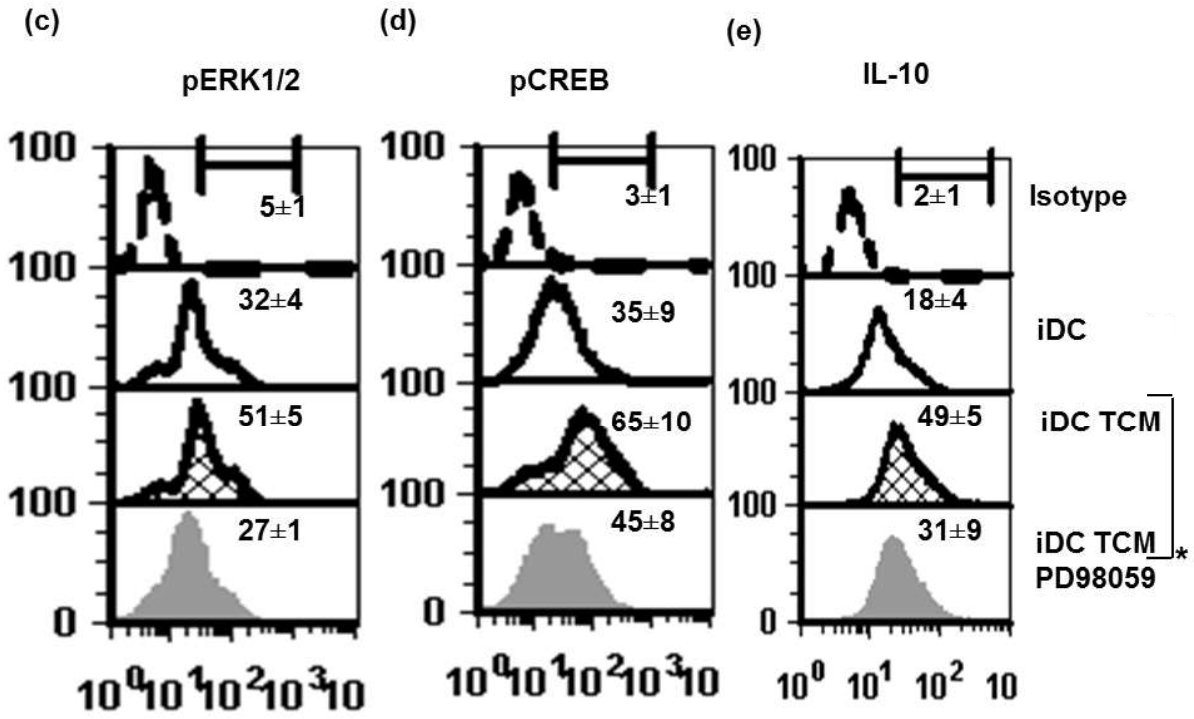


Fig 18. TCM induced IL-10 through ERK/CREB signalling axis: ERK1/2 inhibitor (PD98059) was added to DC TCM culture and expression of (c) phospho ERK1/2, (d) phospho CREB and (e) intracellular IL-10 in iDC, iDC (TCM) and iDC (TCM) treated with PD98059 were analysed through flow cytometry. Number in histogram represents percentage positive cells from three independent experiments (mean ± s.e.m.) (*p< 0.05).

3.1.2 (c) Inhibition of ERK phosphorylation did not abrogate DC dysfunction: As ERK activation was observed to regulate CREB phosphorylation and thus IL-10 induction, effect of ERK inhibition was evaluated on TCM induced DC dysfunction.

PD98059 was added to DC (TCM) culture and expression of maturation markers were analysed on DC. It was observed that though the expression of CD40 and MHCII was downregulated to 35% and 9% respectively in DC (TCM) as compared to 60% and 18% in DC alone, inhibition of ERK phosphorylation did not restore the expression of CD40 (40%) and MHCII (6%) (Fig 19a-b). Intracellular labelling of IL-10 further showed that expression of IL-10 was increased with TCM and decreased with TCM + PD98059 but this decrease in IL-10 could not be translated into restoration of phenotypic maturation of DC (TCM) (Fig 19c).

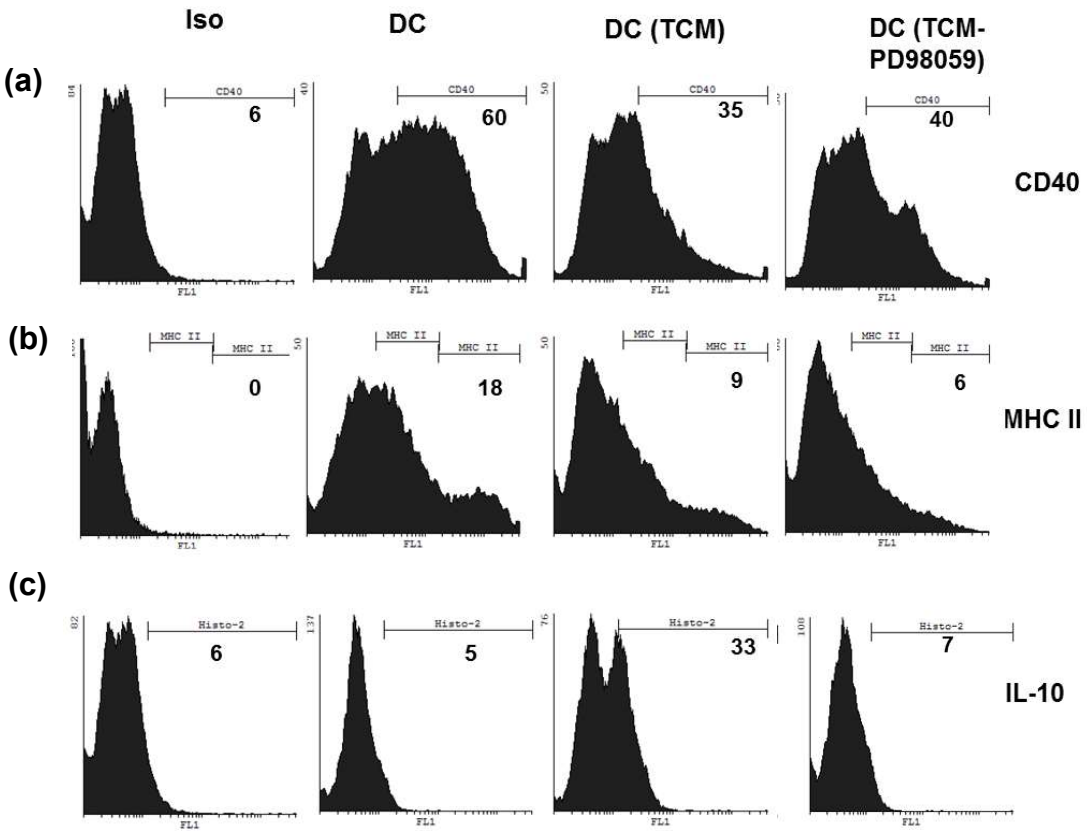


Fig 19: Inhibition of ERK phosphorylation did not abrogate TCM induced decrease in DC maturation markers. DC (TCM) culture was treated with PD98059 from day 1 along with DC control. On day 8, DC, DC (TCM), and DC (TCM-98059) were

labelled for the expression of (a) CD40, (b) MHCII and (c) IL-10 through flow cytometry. Number in histogram is percentage positive cells and data shown is one of the representative of two independent experiments.

3.1.2 (d) Effect of rIL-10 on differentiation and maturation of BMDC:

IL-10 is a potent immunosupressive cytokine and have been shown to negatively regulate the immunogenicity of dendritic cells (*12, 209, 210*). We have also observed that TCM induced secretion of IL-10 in DC (TCM) through ERK-CREB mediated pathway. Inhibition of ERK phosphorylation inhibited CREB phosphorylation and thus IL-10 secretion, however could not mitigate DC dysfunction. Next we explored whether IL-10 was the primary factor responsible for impairment of DC**.**

Exogenous rIL-10 (50 ng/ml) was supplemented either on day 5 (differentiation) or on day 8 (maturation). Expression of maturation markers was analysed both in iDC and mDC. CD40 expression decreased from 52% in iDC to 26% in iDC (TCM), but remained unchanged with 55% in iDC (rIL-10) (Fig 20a). Similarly CD80 and MHCII expression decreased in mDC (TCM) as compared to mDC but no decrease was observed in iDC (rIL-10) (Fig 20b,c). These observations showed that IL-10 alone was inadequate to down regulate expression of maturation markers and did not mimic the effects of TCM. Nonetheless, mDC (IL-10) showed down regulation of maturation markers as compared to control DC and the effects were not to the same extent as TCM (Fig 20 d,e).

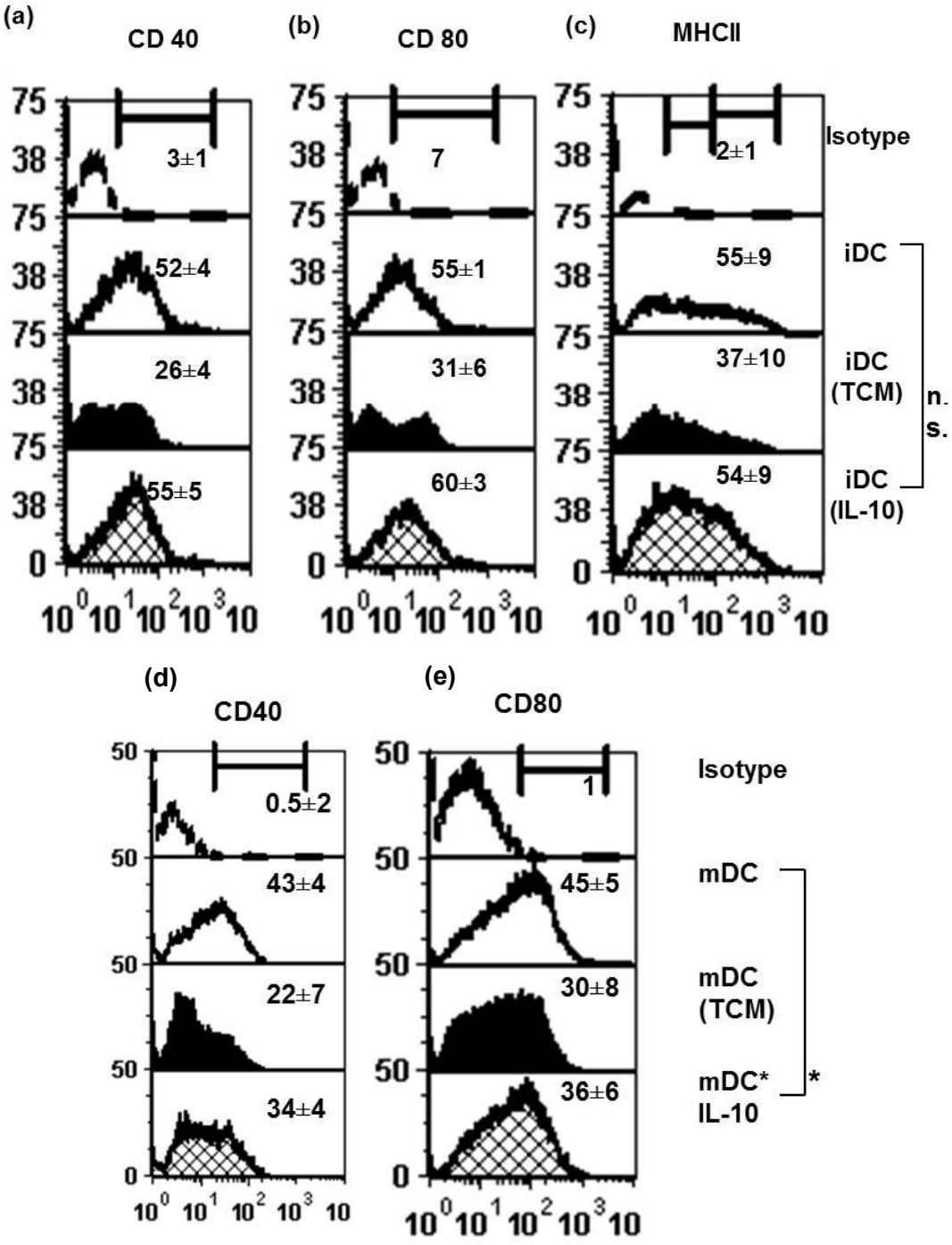


Fig 20: Exogenous IL-10 affected only DC maturation and not DC differentiation: Exogenous recombinant IL-10 (50 ng/ml) was added in DC culture on day 5 and expression of (a) CD40, (b) CD80 and (c) IA/IE in immature DC were analysed by flow

cytometry. rIL-10 was added during maturation and expression of (d) CD40, (e) CD80 were analysed in mDC, mDC (TCM) and mDC (rIL-10). Data shown are histograms from a representative experiment and the values in the histogram are mean ± s.e.m. of percentage positive cells from three experiments (n.s.- not significant, *p < 0.05).

3.1.3 Effect of cancer microenvironment on DC progenitors: The observation that the inhibitory effect of TCM was observed during early stage of DC differentiation suggested that tumor derived factors were affecting the progenitor cells of DC. To further explore the possibility of cancer microenvironment affecting DC progenitors, BMC isolated from tumor bearing mice were analysed for the expression of several lineage specific transcription factors.

(a) BMDC from TBM showed downregulation of phenotypic maturation: BMDC were generated from bone marrow cells of tumor bearing mice following standard protocol. Expression of maturation markers were analysed on BMDC (TBM) in comparison to BMDC control. We observed that expression of CD40, CD86 and MHCII decreased from 60% to 32%, 23% to 12%, 61% to 42% respectively (Fig 21a-c).

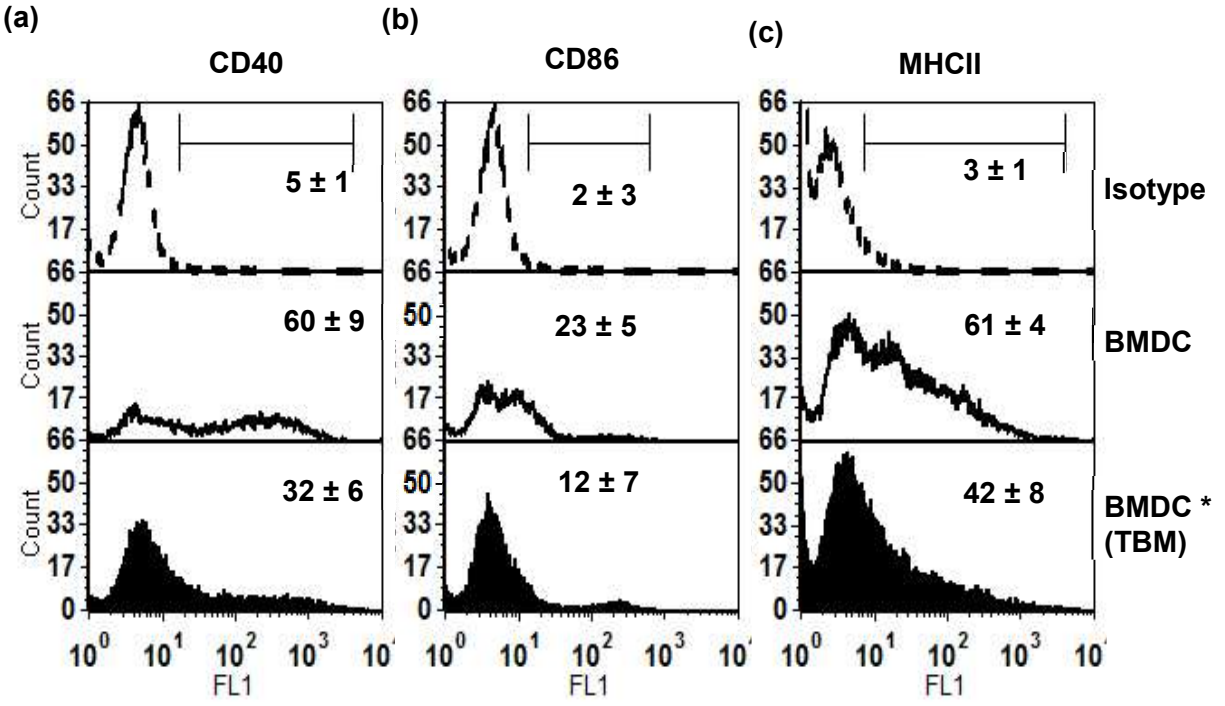


Fig 21: DC (TBM) showed phenotypic and functional immunosuppression. BMC were isolated from TBM and cultured for the differentiation of DC. Expression of (a) CD40, (b) CD86 and (c) MHCII in BMDC of NT and TBM were evaluated using flow cytometry. Values are mean ± s.e.m. of percentage positive cells from three independent experiments (*p<0.05).

(b) BMDC (TBM) showed downregulation of allogenic T cell proliferation capacity: As the BMDC generated from TBM were showing phenotypic suppression of maturation markers, we next examined the allogenic T cell proliferation capacity of DC (TBM). DC and DC (TBM) generated from C57BL/6 (H-2^b) mice were incubated with allogenic T cells from BALB/c mice (H-2^d) and T cell proliferation was measured using ^{3}H-thymidine incorporation. Fig 21d shows that the proliferation of T cells against allogenic antigen

was significantly down regulated when co-cultured with DC (TBM) as compared to DC in DC:T cell ratios of 1:10 and 1:20) ($p<0.05$).

(d)

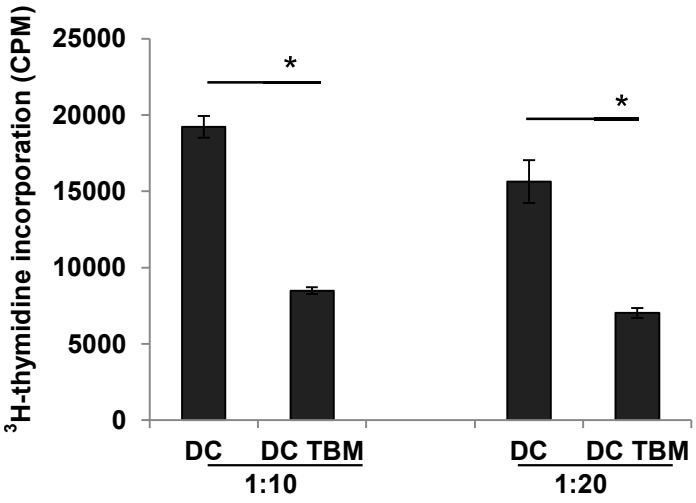
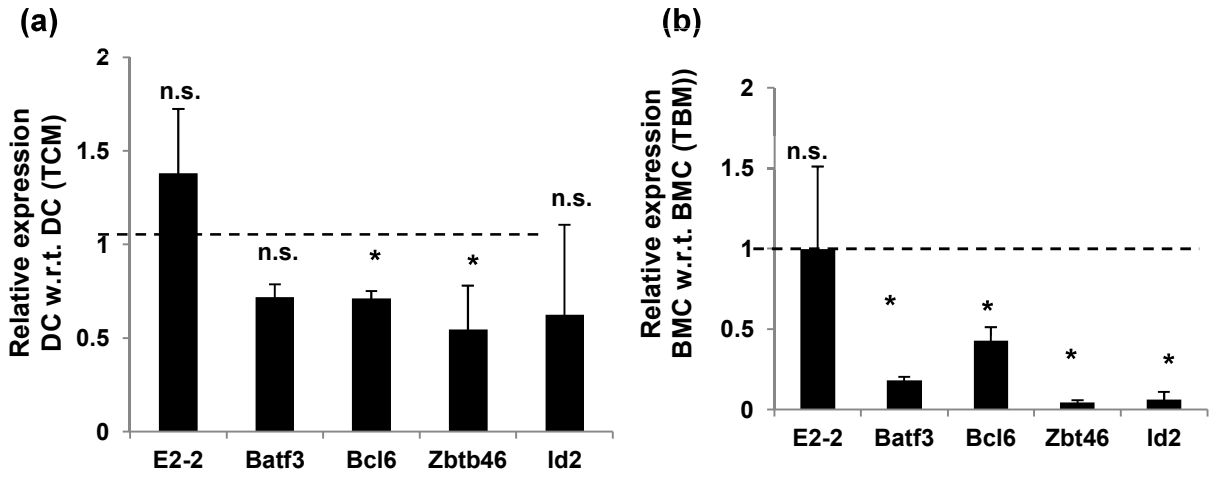


Fig 21: DC (TBM) showed phenotypic and functional immunosuppression. (d) Allogenic T cell proliferation capacity of DC (TBM): Irradiated stimulator DC (H-2^b) and DC (TBM) were co-cultured with allogeneic T cells (H-2^d) from BALB/c mice. DC to T cells ratios of 1:10 and 1:20. T cell proliferation was measured by ^{3}H-thymidine incorporation. Values are mean ± s.e.m. of six replicates. *$p < 0.01$.

(c) Role of lineage specific transcription factors in TCM mediated DC dysfunction: BMDC generated from tumor bearing mice without addition of external TCM also showed phenotypic and functional dysfunction similar to the *in vitro* generated DC(TCM) confirming the hypothesis that tumor microenvironment affected the DC progenitor cells. Different transcription factors have been shown to regulate fate of dendritic cells at progenitor stage and have been used as signature for particular lineages of DC (*119, 122, 125, 126*). In this context, the role of lineage specific transcription factors in cancer induced DC dysfunction was studied.

(i) DC (TCM) and BMC (TBM) showed downregulation of Zbtb46 and Bcl6 transcription factors: Expression of transcription factors Id2, Zbtb46, Bcl6, E2-2 and Batf3 were analysed in DC (TCM) as compared to control DC (Fig 22a) and BMC (TBM) as compared to BMC (NT) (Fig 22b). Expression of Zbtb46 and Bcl6 was consistently downregulated in DC (TCM) and BMC (TBM). Zbtb46 expression is associated with the commitment of CDPs to the cDC lineage only and therefore Zbtb46 serve as a signature marker for cDC (*120*). Expression of Zbtb46 was also monitored by western blot and flow cytometry using specific antibodies. Downregulation in the expression of Zbtb46 in DC (TCM) and BMC (TBM) was confirmed by flow cytometry analysis where expression of Zbtb46 decreased from 32% in DC to 17% in DC (TCM) and 50% in BMC to 35% in BMC (TBM) (Fig 22c, d). Western blot analysis also confirmed Zbtb46 downregulation in DC (TCM) and DC (TBM) (Fig 22e).



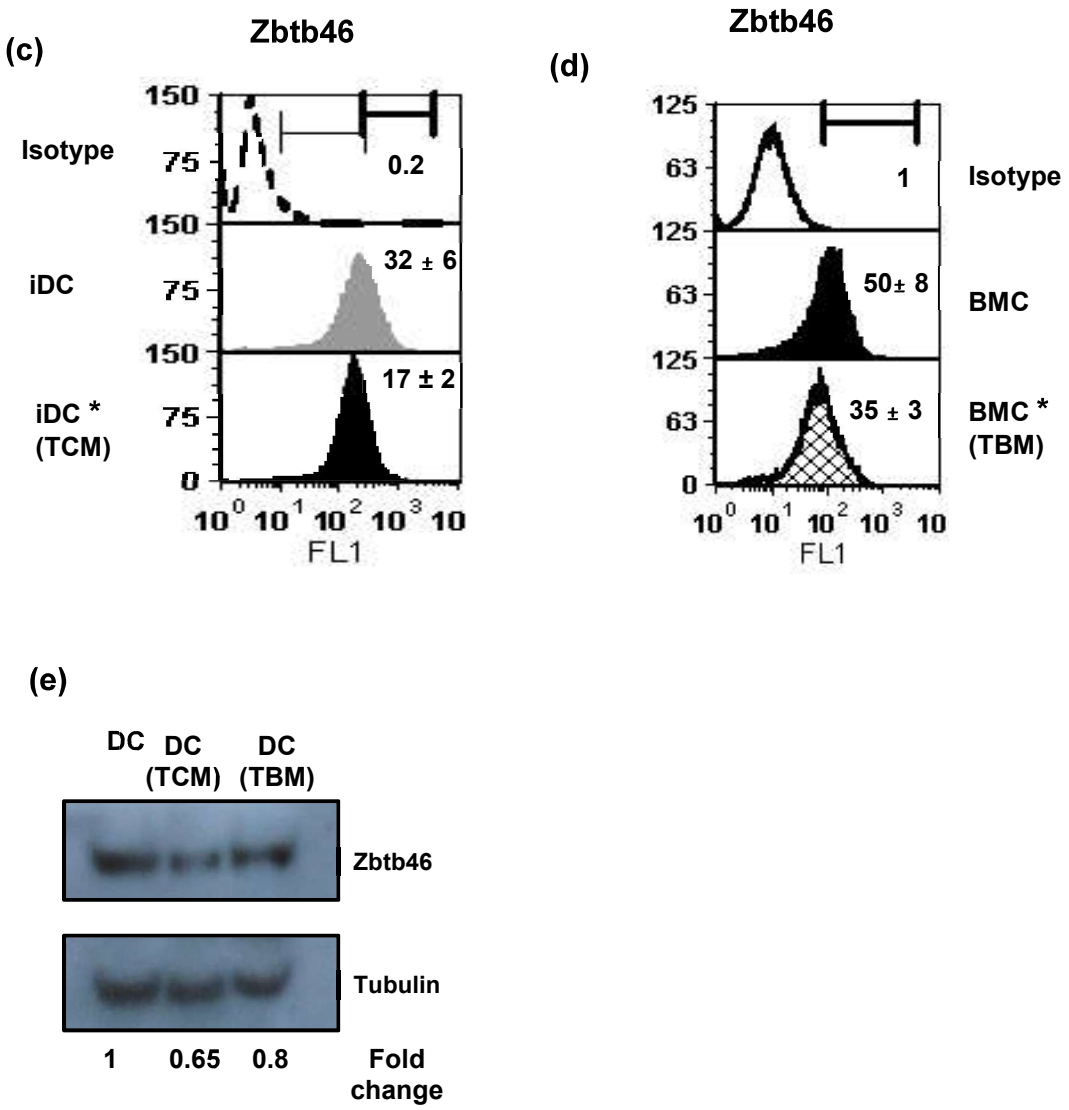


Fig 22: **Expression of lineage specific transcription factors in DC present in tumor microenvironment:** Expression of E2-2, Batf3, Bcl6, Zbtb46 and Id2 in (a) DC (TCM) in comparison to DC and in (b) BMC (TBM) in comparison to BMC quantified using qPCR. REST^{TM} software was used to quantify the relative expression. *p<0.05. (c) Expression of Zbtb46 in DC and DC (TCM) as well as in (d) BMC and BMC (TBM) was quantified by flow cytometry and (e) by western blot. Data shown in (c-d) are histograms from a representative experiment and the values in the histogram are mean ± s.e.m. of percentage positive cells from three experiments (*p<0.05).

(ii) siRNA mediated knockdown of Zbtb46 mimicked effect of TCM on BMDC: BMC (TBM) as well as BMDC (TCM) showed downregulation of transcription factor Zbtb46. In order to explore whether Zbtb46 downregulation has any impact on BMDC immunogenicity, Zbtb46 was knocked down using specific siRNA either on day 0 or day 8. For day 0 knockdown, BMC were incubated with siRNA immediately after isolation and then further cultured with GM-CSF and IL-4 following standard protocol. For day 8 knockdown, control iDC were incubated with Zbtb46 specific siRNA and then further treated with LPS for maturation. Confirmation of knockdown was carried out using intracellular labelling of Zbtb46 (Fig 23a). Expression of maturation markers was analysed after LPS treatment. Expression of CD40 decreased from 60% in mDC control to 23% in mDC (day 0 KD) and 53% in mDC (day 8 KD) (Fig 23b). Expression of CD80 decreased from 60% in mDC to 19% in mDC (day 0 KD) and 50% in mDC (day 8 KD) (Fig 23c). Expression of MHC II followed similar pattern and from 39% in mDC its expression decreased to 16% in mDC (day 0 KD) and 40% in mDC (day 8 KD) (Fig 23d). The pattern of decrease in expression of maturation markers due to Zbtb46 KD in BMDC followed similar pattern as BMDC (TCM) i.e. addition of TCM during differentiation period of BMDC significantly inhibited maturation markers. However TCM addition during maturation only had marginally decreased the maturation markers on BMDC.

In order to see the effect of Zbtb46 knockdown on IL-10 secretion, cell culture supernatant was assessed for IL-10 using ELISA. Though DC (TCM) showed significant upregulation of IL-10 secretion when compared to DC, there was no significant change in IL-10 secretion in Zbtb46 as well as Bcl6 knockdown in either day 0 or day 8 (Fig

23e). This indicated that though induction of IL-10 and downregulation of Zbtb46 was regulated by tumor derived factors, these two events were probably independent of each other.

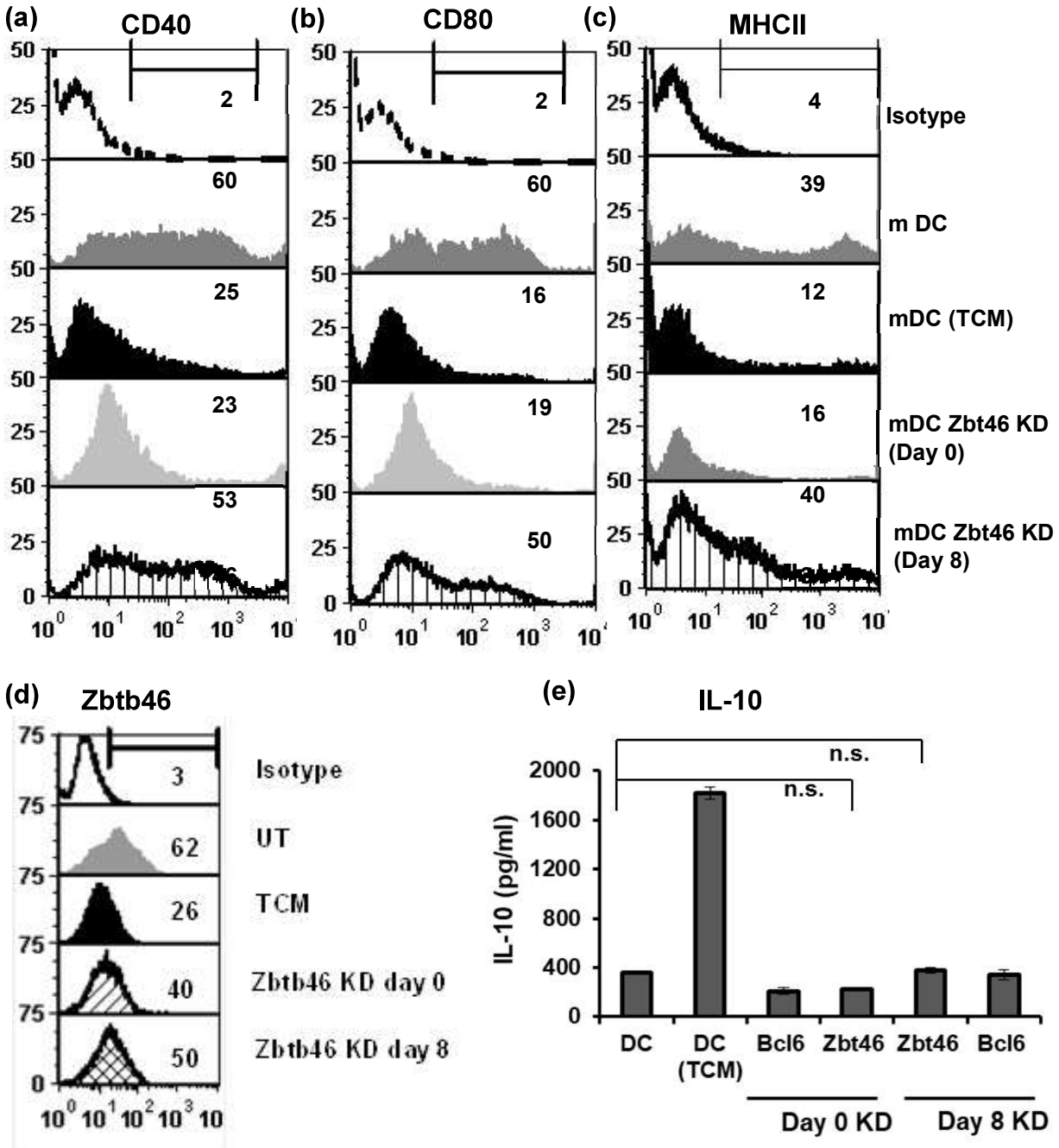


Fig 23: Zbtb46 knockdown in DC mimicked TCM induced DC immunosuppression. Expression of (a) CD40, (b) CD80 (c) MHCII in BMC Zbtb46 KD:

Day 0 and BMC Zbtb46KD: Day8 as analyzed by flow cytometry. Histograms are representative of 2 independent experiments. (d) Knockdown of Zbtb46 on day 0 and 8 were validated on day 8 using flow cytometry (data shown here is representative from two independent experiments). (e) IL-10 was quantified in knockdown DC supernatant using ELISA. Data shown here is mean ± s.e.m. from triplicates (n.s: Not significant).

3.1.4 Role of prostaglandin in TCM induced suppression of DC differentiation.

Tumor derived molecules had induced secretion of IL-10 in DC through ERK/CREB pathway and also downregulated Zbtb46 expression in DC. It was further observed that rIL-10 alone was not sufficient to induce dysfunction of DC as TCM, while knockdown of Zbtb46 mimicked the effects of TCM. One of the candidate upstream molecules that induced IL-10 through a cAMP/PKA/CREB mediated pathway in macrophages is PGE2 (*179*). In this context, role of prostaglandin was probed in DC (TCM) dysfunction.

3.1.4 (a) Tumor derived prostanoids supressed Zbtb46 mediated DC differentiation.

To identify if PGE2 was the tumor derived factor responsible for impairment in DC function, EL4 cells were cultured in presence of NS-398, a selective COX-2 inhibitor. NS-398 itself was not cytotoxic to EL4 cells at 2, 4 and 10 μM concentration (Fig 24a). TCM was collected from NS-398 treated EL4 cells and differentiation of DC was carried out with TCM as well as TCM (NS-398) followed by assessment of maturation markers. The percentage of CD40 positive cells down regulated from 55% to 35% by TCM was

restored to 57% with TCM (NS-398). Similarly TCM induced suppression of CD80 and MHC II positive was restored to control levels with TCM (NS-398) (Fig 24b).

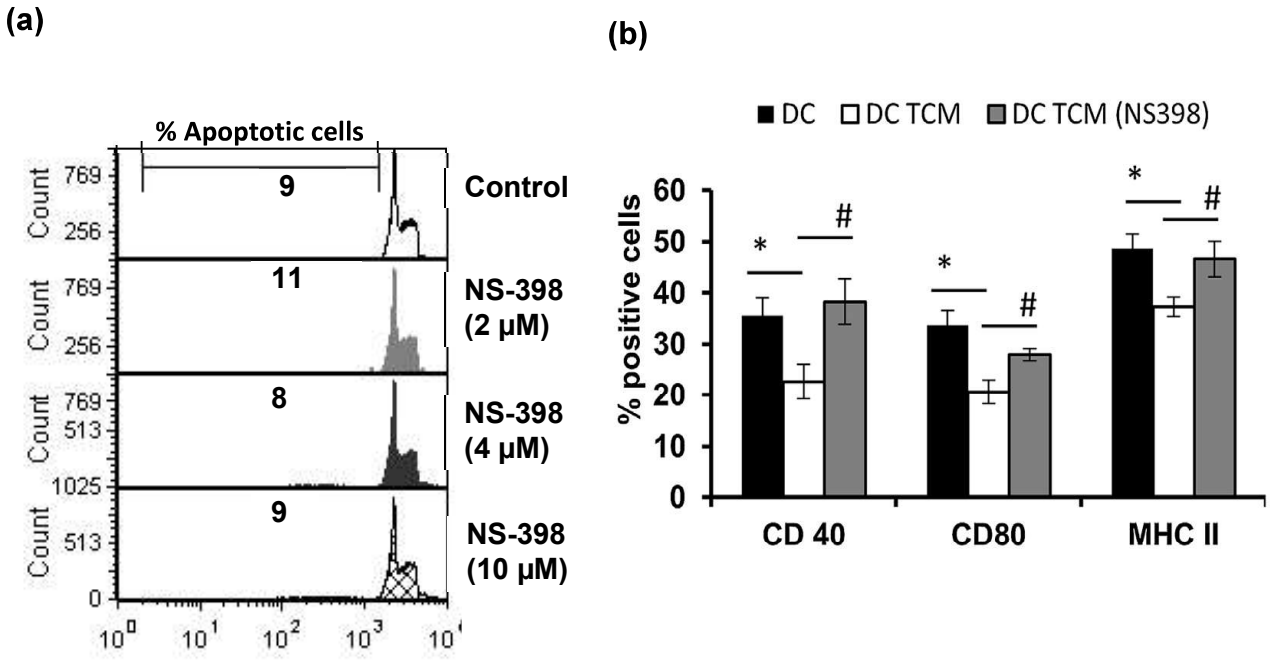


Fig 24: **Tumor derived prostanoids suppressed Zbtb46 mediated DC differentiation: (**a) NS-398 did not have cytotoxic effects on EL4 cells: EL4 cells were treated with different concentration of NS-398 (2, 4 and 10 µM) and cell cycle analysis were done using PI staining to evaluate NS-398 cytotoxicity. Pre G1 population represent percentage of apoptotic cells. Histograms are representative of 2 independent experiments. (b) Expression of CD40, CD80 and MHCII in DC, DC (TCM) and DC (TCM: NS 398) as determined by flow cytometry. Values in the histogram are mean ± s.e.m. of percentage positive cells from three experiments (*$p < 0.05$,#$p < 0.05$).

3.1.4 (b) NS-398 did not directly affect DC differentiation: NS-398 treatment to EL4 cells prevented the TCM induced downregulation of maturation markers on DC, suggested that the inhibition of tumor derived PGE2 was important for averting TCM induced DC dysfunction. To rule out the possibility that NS-398 present in TCM could have had a direct effect on DC immunogenicity, NS-398 was added into the DC culture in absence of TCM and expression of maturation markers was analysed. Expression of CD40 was downregulated to 21% in DC (TCM) as compared to 59% in DC and it was 24% in DC NS-398 (TCM) (Fig 25a). Expression of CD80 was downregulated to 25% in DC (TCM) as compared to DC and it was 34% in DC NS-398 (TCM) (Fig 25b). Expression of MHC also followed similar pattern and it was 48%, 34% and 38% in DC, DC (TCM) and DC NS398 (TCM) respectively (Fig 25c). These results indicated that the effect observed earlier (Fig 24b) was due to inhibition of COX-2 in tumor cells by NS-398.

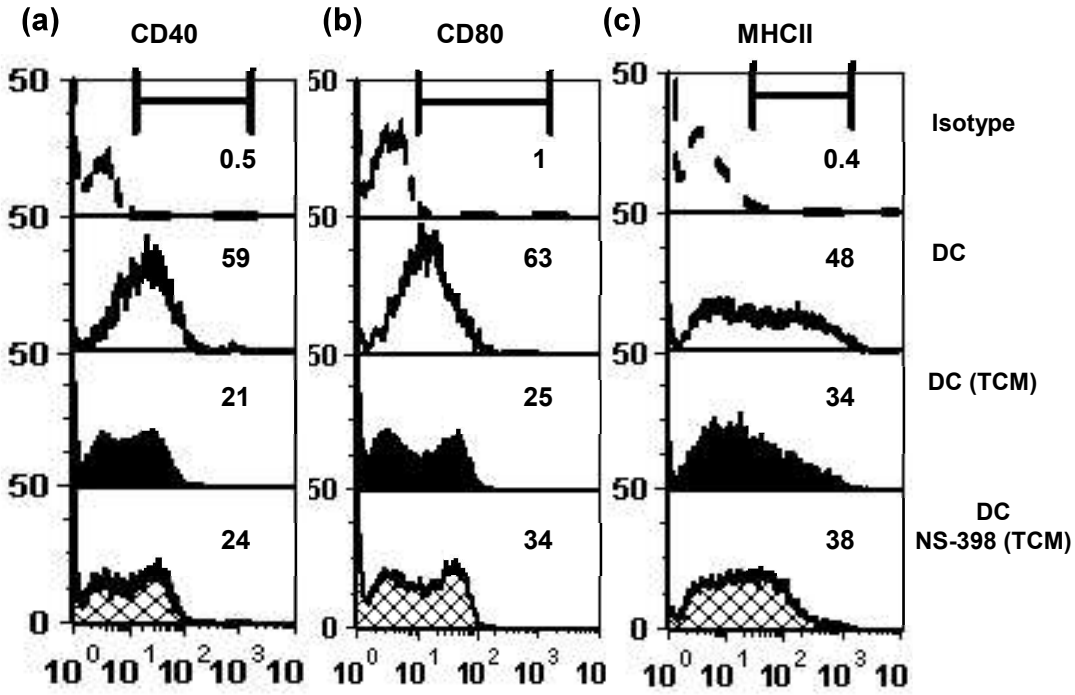


Fig 25: Inhibition of COX-2 in dendritic cells did not rescue TCM induced suppression of maturation : NS-398 (10 μM) was added into DC (TCM) culture and expression of (a) CD40, (b) CD80 and (c) MHCII was analysed using flow cytometry. Data shown are histograms from a representative experiment from two independent experiments. Numbers reflect the percentage of positive cells in each group.

3.1.4 (c) Exogenous PGE2 downregulated DC maturation and Zbtb46 expression: To further confirm that PGE2 derived from cancer cells played a crucial role in Zbtb46 downregulation and subsequently DC dysfunction, BMC from control mice or BMC neutralised with EP2 antibody against PGE2 receptor were incubated with PGE2 for 48 h to analyse the expression of Zbtb46 using flow cytometry. PGE2 down regulated the $Zbtb46^{high}$ population of BMC from 35% to 19% which was restored significantly ($p<0.05$) to 30% with EP2 blocking antibody (Fig 26a). In comparison, incubation of BMC with TCM down regulated the $Zbtb46^{high}$ population of BMC from 34% to 25% within 48 h. Further, DC were also differentiated in presence of rPGE2 from day 0 and expression of maturation markers was quantified using flow cytometry. Expression of CD40 was downregulated to 15% in mDC (PGE2) as compared to 51% in mDC (Fig 26b) and expression of MHCII was downregulated to 29% in mDC (PGE2) from 49% in mDC (Fig 26c). These results indicated that, though PGE2 is a major component of tumor derived factors, other prostanoids also could play a role and inhibition of COX-2 is more effective to avert the effect of TCM on DC differentiation.

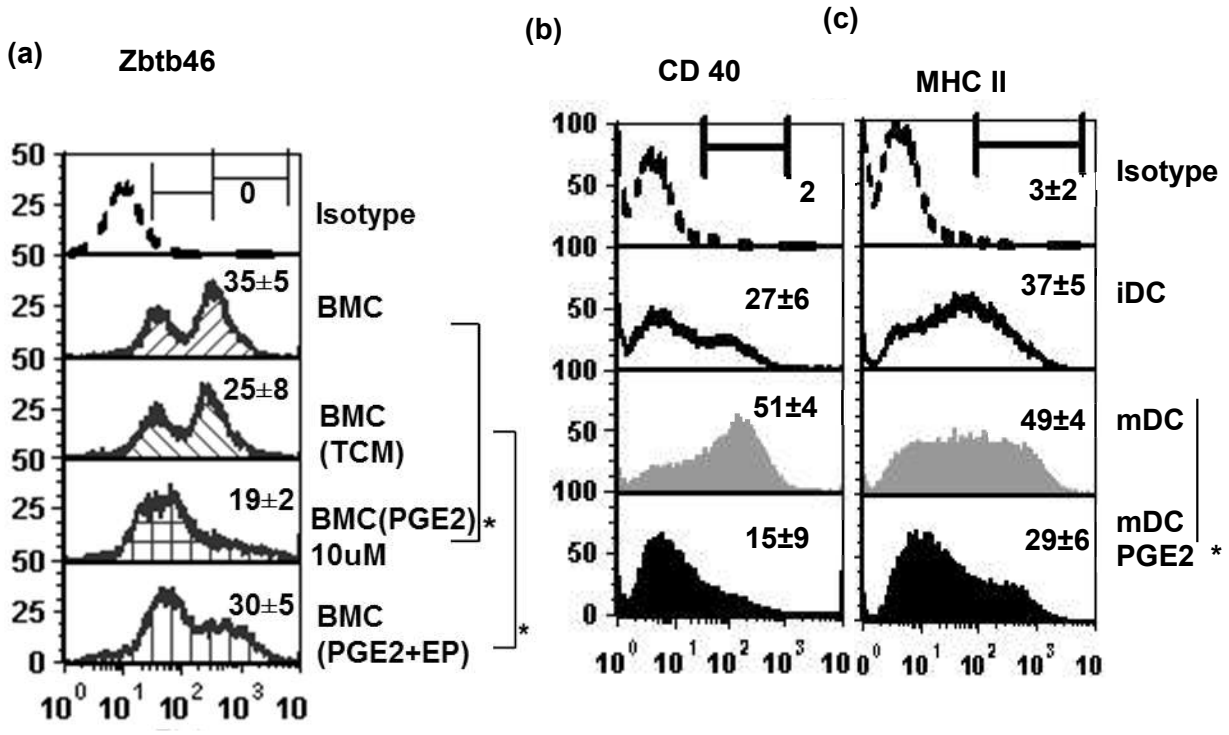


Fig 26: Exogenous PGE2 induced DC dysfunction through downregulation of Zbtb46. (a) Expression of Zbtb46 in BMC incubated with PGE2 (10 μM) alone or with pre-treatment of EP2 blocking antibody (5 μg/ml) for 48 h. Expression of (b) CD 40 and (c) MHC II in DC supplemented with exogenous PGE2 (10 μM) on day 0 of DC culture. Data shown are histograms from a representative experiment and the values in the histogram are mean ± s.e.m. of percentage positive cells from three experiments. *p < 0.05.

3.1.4 (d) Effect of NS-398 on DC dysfunction and tumor burden in mouse lymphoma model: The observation that COX-2 inhibitor NS-398 could avert the TCM induced DC dysfunction *in vitro*, led us to investigate whether this effect of NS-398 can be carried forward under *in vivo* condition and restoration of DC function could possibly

affect the tumor burden in a mouse lymphoma model. For this purpose, tumor bearing mice (TBM) model were generated with s.c. injected EL4 cells and TBM were segregated into 3 groups: TBM (control), TBM (vehicle) and TBM (NS-398). TBM (vehicle) and TBM (NS-398) were treated with DMSO and NS-398 respectively.

(i) NS-398 treatment decreased the serum PGE2 level: Mice were sacrificed on day 19 and serum was collected from all the groups. The level of PGE2 was quantified in serum using ELISA. There was a 2 fold increase in serum PGE2 levels in TBM (control) and TBM (vehicle) as compared to control mice with no tumor (NT). Following treatment, TBM (NS-398) had lower PGE2 levels and was comparable to NT ($p<0.05$; Fig.27a).

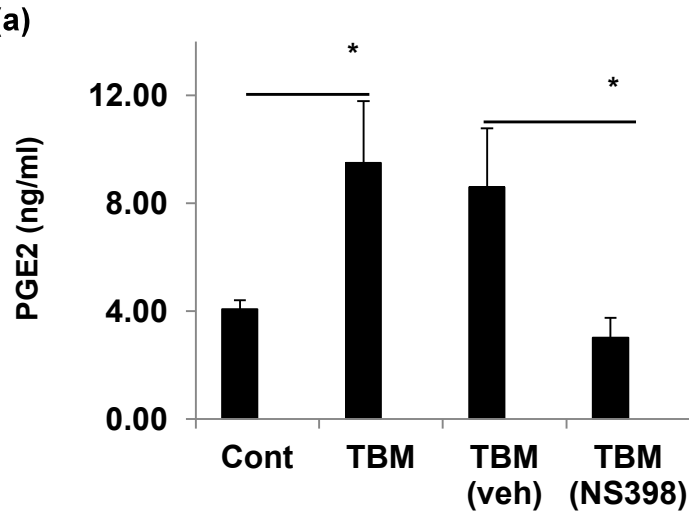


Fig 27: COX-2 inhibitor NS-398 treatment resulted in immunocompetent DC and reduced tumor burden in lymphoma model. (a) Mice serum was collected on day 19 of tumor injection and PGE2 level was quantified using ELISA. Values are mean ± s.e.m. of triplicates(*$p < 0.05$).

(ii) NS-398 treatment restored Zbtb46 expression and immunogenicity of splenic DC from TBM: The expression of Zbtb46 was evaluated in BMC of these different treatment groups. Zbtb46 was down regulated in TBM (control) (38%) and TBM

(vehicle) (40%) as compared to NT BMC (60%) and expression was restored to 65% in BMC from TBM (NS-398) (Fig 27b).

$CD11c^+$ splenic DC were isolated from all the groups using magnetic microbeads and the maturation status of $CD11c^+$ splenic DC from mice of different treatment groups was analyzed. CD40 positive cells decreased from 43% in splenic DC of NT mice to 28% in TBM (control), 30% in TBM (vehicle) and were restored to 42% in TBM (NS-398) (Fig 27c). Similarly, the expression of CD80 decreased from 32% in splenic DC of NT mice to 18% in TBM (control), 19% in TBM (vehicle) and were restored to 24% in TBM (NS-398) (Fig 27d) and expression of MHC II also followed similar pattern and its expression was 56%, 34%, 34% and 55% in NT, TBM (control), TBM (vehicle) and TBM (NS-398) respectively (Fig 27e).

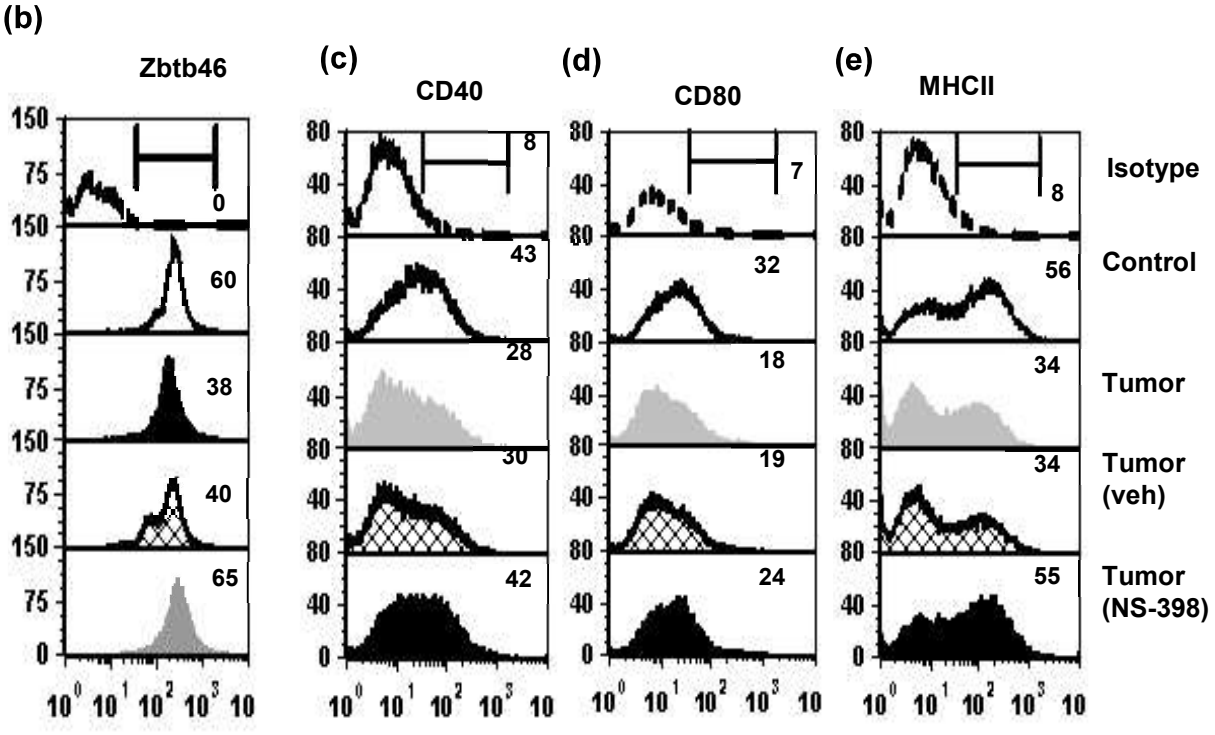


Fig 27: **COX-2 inhibitor NS-398 treatment resulted in immunocompetent DC and reduced tumor burden in lymphoma model.** NS-398 treatment restored Zbtb46 level

in BMC of TBM and resulted in immunocompetent DC. Expression of (b) Zbtb46 in BMC (c) CD40 (d) CD80 and (e) MHCII in $CD11c^+$ splenic DC of C57BL/6 mice from all treatment groups. Histograms are representative of 2 independent experiments.

Further to assess the functional competency of splenic DC, a mixed leucocyte reaction (MLR) was setup with splenic DC from different groups as stimulator and $CD4^+$ T cells from BALB/c mice as responder. T cells were incubated with γ-irradiated splenic DC in 1:4 ratios of DC:T cells. Splenic DC from TBM (control) and TBM (vehicle) showed significant down regulation of proliferative response in allogenic T cells when compare to splenic DC from NT mice ($p<0.05$). However NS-398 treated splenic DC showed augmented proliferative responses in allogenic T cells as compared to TBM (control) and TBM (vehicle) ($p<0.05$) (Fig 27f).

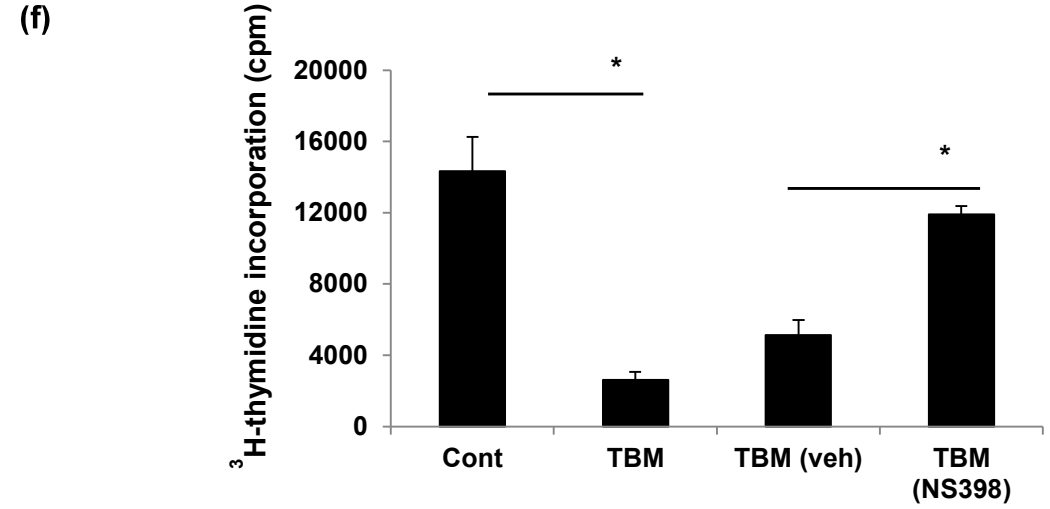


Fig 27: COX-2 inhibitor NS-398 treatment resulted in immunocompetent DC and reduced tumor burden in lymphoma model. NS-398 treatment restored functional competency of DC. (f) Allogenic T cell proliferation capacity was assessed by MLR. sDC from all groups were co-cultured with allogeneic T cells from BALB/c mice in 1:4 ratio. T

cell proliferation was measured by ^{3}H-Thymidine incorporation. Values are mean ± s.e.m. of six replicates (*p < 0.05).

(iii) NS-398 treatment reduced tumor burden in TBM: Since NS-398 was not cytotoxic to EL-4 lymphoma cells, the next question was whether this augmented DC function will be sufficient to boost anti-tumor immune responses resulting in decreased tumor burden? Tumor volume was measured from day 9 onwards. As seen in Fig 27g, on day 17, tumor burden in TBM (NS-398) was reduced by 54% when compared to TBM (control) (p=0.008) and 52% when compared to TBM (vehicle) (p=0.01). Fig 27h shows the dissected tumors from each group on day 19.

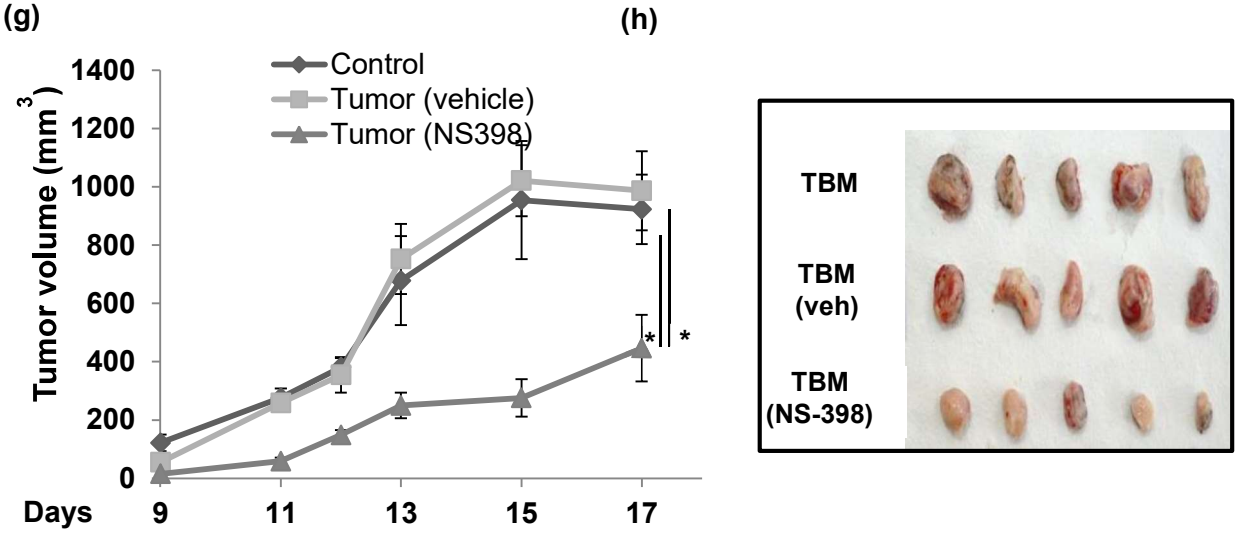


Fig 27: NS-398 treatment reduced tumor burden in TBM. (g) Tumor volume of all the groups was monitored from day 9 onwards till day 17. It was calculated as (small diameter)^{2} X large diameter X 0.53. (h) Tumors were dissected on day 19 (n = 5) in C57BL/6 mice. Representative data from three independent experiments are shown (*p < 0.05).

3.1.4 (e) Effect of NS-398 treatment in tumor bearing immunodeficient mice (SCID):

NS-398 treatment in immuno-competent TBM had shown restoration of phenotypic and functional immunogenicity of DC and subsequent reduction in tumor burden. This observation suggested that anti-tumor effect of NS-398 was through dendritic cells. To further confirm this hypothesis, anti-tumor effect of NS-398 was tested in EL4 lymphoma bearing immuno-incompetent SCID (severe combined immunodeficiency) mice.

(i) Confirmation of absence of adaptive immune response in SCID mice: TBM-SCID were segregated into TBM (control), TBM (vehicle) and TBM (NS-398). Expression of CD3 and CD19 were evaluated in SCID mice to verify the immunodeficiency along with BALB/c splenocytes as positive control. Fig 28a and b showed that in BALB/c, 43% and 40% cells were positive for the expression of CD3 and CD19 respectively but in all other SCID mice groups expression of CD3 and CD19 were severely downregulated closer to the isotype control.

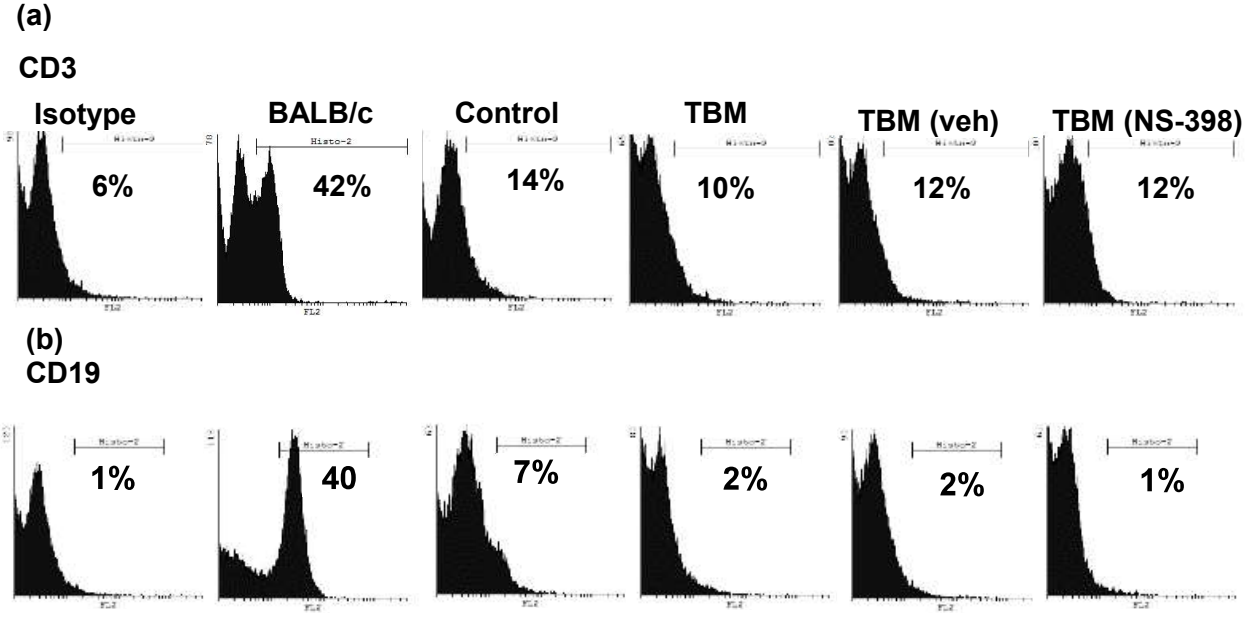
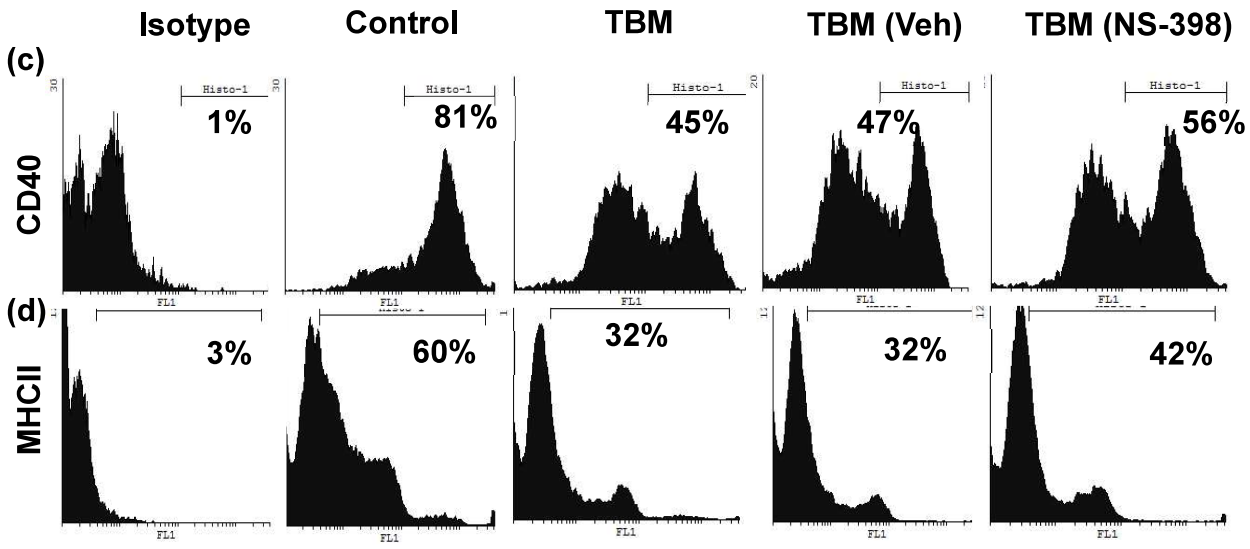


Fig 28: Effect of NS-398 treatment in lymphoma SCID mice model. Expression of (a) CD3 and (b) CD19 were assessed in lymphocytes isolated from all treatment groups of SCID mice and BALB/c mice as positive control. Number in each histogram represents percentage positive cells from single experiment.

(ii) NS-398 treatment marginally restored DC maturation and had no effect on tumor burden: To assess the effect of NS-398 on DC in TBM-SCID, $CD11c^{+}$ DC were magnetically isolated and expression of maturation markers was quantified using flow cytometry. Expression of CD40 was downregulated to 45% in TBM and 47% in TBM (vehicle) compared to 81% in control mice and it was upregulated to 56% in TBM (NS-398) (Fig 28c). Similarly expression of MHC II was downregulated to 32% in each of TBM and TBM (vehicle) as compared to 60% in control and it was 42% in TBM (NS-398) (Fig 28d).

Tumor volume was monitored from day 12 to day 22 and there was no significant difference in tumor size between TBM, TBM (vehicle) and TBM (NS-398) (Fig 28e)



(e)

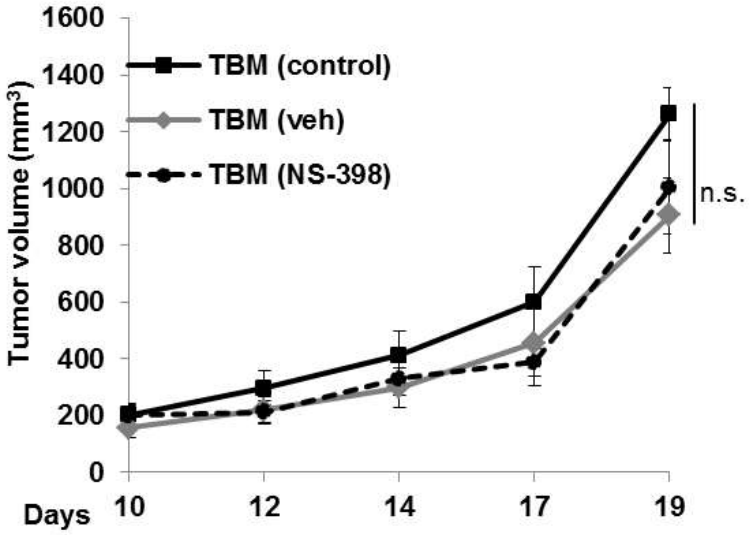


Fig 28: **Effect of NS-398 treatment in lymphoma SCID mice model:** Splenic cells isolated from different treatment groups of SCID mice and expression of (c) CD40 and (d) MHCII was analyzed using flow cytometry. Experiments were carried out in triplicate. Number in histogram represent percentage positive cells. (e) Tumor volume was monitored from day 10 onwards till day 19 (n.s.-non significant).

3.1.4 (f) Effect of NS-398 along with chemotherapeutic drug camptothecin (CPT) in TBM:

Treatment with NS-398 had resulted in restoration of DC function in TBM and tumor burden was also reduced significantly. Role of DC was further confirmed when treatment of NS-398 did not reduce the tumor burden in SCID-TBM. Interestingly, in SCID mice, though there was increased DC immunogenicity because of NS-398 treatment, there was no effect on tumor volume possibly due to the lack of effector T cells. This anti tumor effect of NS-398 was achieved despite the fact that there was no

direct cytotoxic effect of NS-398 on EL4 cells *in vitro*. The effect of NS-398 was further tested with a cytotoxic drug camptothecin (CPT) in mice lymphoma model for potential synergistic anticancer effect of NS-398 and CPT. Mice were segregated into 4 groups: TBM (control), TBM (NS-398), TBM (CPT) and TBM (NS-398+CPT) along with control. TBM (NS-398) mice and TBM (NS-398+CPT) mice were injected with NS-398 from day 1 to day 12, while TBM (CPT) and TBM (NS-398+CPT) mice was injected with CPT on days 3, 6, 9 and 12. Mice were sacrificed on day 17 and various parameters were analyzed.

(i) Effect of NS-398 with CPT on splenic DC: Splenic DC were isolated using magnetic microbeads and phenotypic maturation was evaluated. Expression of CD40 was decreased from 60% in control to 43% in TBM and restored to 51% in TBM (CPT), 51% in TBM (NS-398) and 58% in (NS+CPT) (Fig 29a). Similarly expression of CD80 was 70%, 40%, 46%, 56%, 52% in control, TBM, TBM (CPT), TBM (NS-398) and TBM (NS+CPT) (Fig 29b). Expression of MHC II decreased to 43% in TBM as compared to 58% in control and there was no significant improvement in TBM (CPT) with 48%. However, MHC II expression increased to 54% in TBM (NS-398) and 65% in TBM (NS+CPT) (Fig 29c). Maturation status of sDC showed that unlike NS-398 treatment, CPT treatment did not significantly rescue the maturation status of sDC.

(ii) NS-398 along with cytotoxic drug CPT significantly improved anti tumor response as compared to CPT alone: Tumor volume was significantly reduced in TBM (NS-398) ($p<0.05$) and TBM (CPT) ($p=0.007$) when compared to TBM control. Tumor volume was further reduced significantly in TBM (NS-398+CPT) when compared

to TBM (NS-398) (p=0.004) as well as when compared to TBM (CPT) (p=0.011) (Fig 29d).

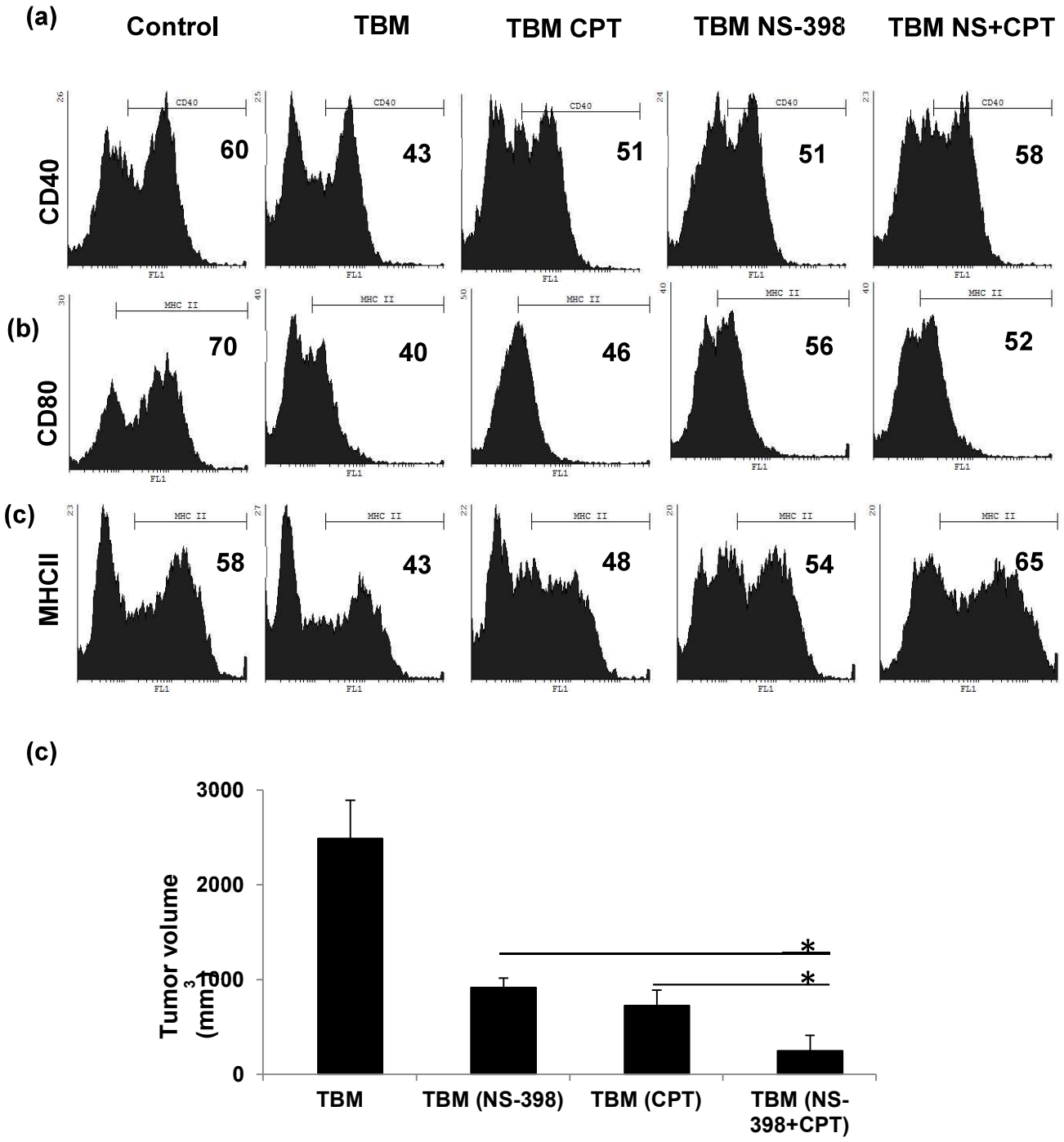


Fig 29: Effect of NS-398 treatment along with CPT in lymphoma model: Tumor

bearing mice were segregated into 4 groups based on their treatment, TBM, TBM (CPT), TBM (NS-398) and TBM (CPT +NS-398). NS-398 was injected from day 1 to day 12 and CPT treatment was given on days 3, 6, 9 and 12. Mice were sacrificed on day 17 and expression of (a) CD40, (b) CD80, (c) MHCII was evaluated using flow cytometry. Number in histogram represents percentage positive cells. (d) Tumor volume of all the groups as measured on day 17 (*$p<0.05$).

RESULT 3.2: Differential expression of miRNA in cancer induced DC dysfunction

3.2.1 Quality control analysis: RNA sequencing was carried out with 2 biological replicates for each sample, iDC, iDC TCM, mDC, mDC TCM, BMC NT and BMC TBM. QC data table of the library were as tabulated as follows:

Table 7: QC data table of samples.

Sample	Raw Reads[1]	Clean Reads[2]	Raw Bases[3] (GB)	Clean Bases[4] (GB)	Error Rate[5] (%)	Q20[6] (%)	Q30[6] (%)
iDC_1	39994302	39879331	2	1.994	0.01	97.36	94.22
iDC_2	39649113	39544924	1.982	1.977	0.01	97.35	94.17
iDC TCM_1	44746521	44564907	2.237	2.228	0.01	97.15	93.86
iDC TCM_2	47130921	47024495	2.357	2.351	0.01	97.31	93.91
mDC_1	41497948	41372629	2.075	2.069	0.01	97.27	93.98
mDC_2	42409919	42308905	2.12	2.115	0.01	97.41	94.26
mDC TCM_1	53872129	53721783	2.694	2.686	0.01	97.3	94.01
mDC TCM_2	42169884	42025660	2.108	2.101	0.01	96.91	93.34
BMC NT_1	42100282	42010298	2.105	2.101	0.01	97.46	94.27
BMC NT_2	41028301	40928015	2.051	2.046	0.01	97.53	94.57

BMC TBM_1	39426633	39291299	1.971	1.965	0.01	97.17	93.75
BMC TBM_2	40250994	40161915	2.013	2.008	0.01	97.21	93.78

[1]Raw reads: contains sequence data along with 5' primer contaminants, oversized insertion, low quality reads, poly A tags and small tags etc.

[2]Clean reads: Sequence data after trimming 3' primer sequence and removal of insert tag and 5' primer contaminants.

[3]Raw bases: (Number of sequences) * (sequence length)

[4]Clean bases: (Number of sequences) * (sequence length)

[5]Error rate: base error rate.

[6]Q20, Q30: (Base number of Phil's Read Editor (Phred) value > 20(> 30)) / (Total base number).

3.2.2 Alignment summary: Differential expression of known and novel miRNA was calculated using miRDeep2 pipeline. Based on miRDeep2 data, the alignment and assignment summery is as follows:

Table 8: Alignment and assignment report.

Sample	Processed Reads[1]	Aligned Reads[2]	Failed to align[3]	Assigned reads[4]	No. of identified entities[5]
iDC_1	11058853	8261363 (74.70%)	2797490 (25.30%)	3840229 (34.7%)	737
iDC_2	9793008	8223341 (83.97%)	1569667 (16.03%)	4071207 (41.6%)	740
iDC TCM_1	12880809	10675084 (82.88%)	2205725 (17.12%)	3823916 (29.7%)	698
iDC TCM_2	13245585	11275542 (85.13%)	1970043 (14.87%)	5878188 (44.4%)	744
mDC_1	8872715	7744784 (87.29%)	1127931 (12.71%)	3204786 (36.1%)	728
mDC_2	12989276	10890981 (83.85%)	2098295 (16.15%)	4859483 (37.4%)	835
mDC TCM_1	14967761	12342938 (82.46%)	2624823 (17.54%)	5608413 (37.5%)	803
mDC	11040003	9317601	1722402	3642554 (33.0%)	724

TCM_2		(84.40%)	(15.60%)		
BMC NT_1	12547019	10599635 (84.48%)	1947384 (15.52%)	5661542 (45.1%)	899
BMC NT_2	11375332	6822945 (59.98%)	4552387 (40.02%)	3366663 (29.6%)	762
BMC TBM_1	12716582	9408631 (73.99%)	3307951 (26.01%)	4023916 (31.6%)	793
BMC TBM_2	11622206	8941498 (76.93%)	2680708 (23.07%)	4563982 (39.3%)	867

[1]Processed read: Number of reads which has been processed under miRNA Deep2 module for alignment.

[2]Aligned reads: Reads which have found complementary position on 'reference Genome'.

[3]Failed to aligned reads: Reads which did not find position on 'reference genome'.

[4]Assigned reads: Reads matching annotated miRNA sequence.

Comparisons were carried out between the following pairs.

(1) iDC vs iDC TCM.

(2) iDC vs mDC.

(3) BMC NT vs BMC TBM.

3.2.3 Differential miRNA expression analysis. Based on the assigned reads, expression profile of different miRNA were evaluated between different groups. We have analyzed differential miRNA expression between iDC and iDC (TCM), iDC and mDC and between NT and TBM.

(a) iDC vs iDC TCM: The RNA sequencing data of iDC and iDC TCM were compared and it was observed that 9 total known miRNAs were significantly differentially regulated in iDC TCM as compared to iDC ($p<0.05$). This included 5 miRNAs that were upregulated significantly and 4 miRNAs that were down regulated significantly.

Table 9: List of miRNAs of significant differential expression between iDC and iDC (TCM).

miRNA ID	Log2 Fold Change	p value	Up/Down
mmu-miR-155-3p	2.466426602	2.2454 E-09	Up
mmu-miR-146a-5p	1.843318738	1.6052 E-05	Up
mmu-miR-132-3p	1.924402368	0.000126154	Up
mmu-miR-155-5p	1.675886607	0.000752963	Up
mmu-miR-129-5p	1.955189861	0.000864076	Up
mmu-miR-504-5p	-2.220651168	3.0201 E-06	Down
mmu-miR-365-2-5p	-1.939432193	2.56675 E-05	Down

mmu-miR-365-1-5p	-1.656432382	0.000193278	Down
mmu-miR-187-3p	-1.600568658	0.00037404	Down
mmu-miR-142a	-1.643410521	0.0101245	Down

(b) iDC vs mDC : Based on the RNA sequencing data, a total of 29 miRNAs were found to be differentially regulated including 3 novel miRNA which have not been annotated. These three miRNAs were located on chromosomes 13, 18 and 4.

Table 10: List of miRNAs of significant differential expression between iDC and mDC.

miRNA ID	**Log2 Fold Change**	**P value**	**Regulation**
chr13:107121703..107121790:+	-11.75514565	7.3694E-68	Down
chr18:85698099..85698171:-	9.255152295	4.95333E-36	Up
chr4:132884366..132884421:-	-8.481394972	3.42556E-28	Down
mmu-miR-504-5p	-2.736265354	1.00898E-13	Down
mmu-miR-378d	-2.307857638	3.15636E-09	Down
mmu-miR-365-2-5p	-2.45873437	8.7235E-09	Down
mmu-miR-365-1-5p	-2.494729398	2.318E-07	Down
mmu-miR-5107-5p	-1.706348609	5.2301E-06	Down
mmu-miR-378c	-1.627718711	6.81382E-06	Down
mmu-miR-187-3p	-2.101391855	1.28675E-05	Down

mmu-miR-378a-3p	-1.553920005	1.46913E-05	Down
mmu-miR-187-5p	-3.151428991	2.90348E-05	Down
mmu-miR-10b-5p	-2.829710157	5.42023E-05	Down
mmu-miR-143-5p	-4.000501735	6.53469E-05	Down
mmu-miR-365-3p	-1.422784614	8.4139E-05	Down
mmu-miR-192-5p	-2.584117345	0.000170022	Down
mmu-miR-145a-5p	-2.492093589	0.000274482	Down
mmu-miR-145a-3p	-3.662182926	0.00028656	Down
mmu-miR-383-5p	-3.355088563	0.00042787	Down
mmu-miR-126a-5p	-3.471793727	0.000663787	Down
mmu-miR-429-3p	-3.268986506	0.001008255	Down
mmu-miR-203-3p	-1.798252542	0.001517272	Down
mmu-miR-139-3p	-1.359116336	0.00161661	Down
mmu-miR-100-5p	-1.794016308	0.002732547	Down
mmu-miR-155-3p	2.900934826	2.99419E-16	Up
mmu-miR-129-5p	2.933258692	8.02622E-11	Up
mmu-miR-146a-5p	1.426754092	2.53745E-05	Up
mmu-miR-132-5p	2.254740331	5.4117E-05	Up
mmu-miR-155-5p	1.203892558	0.000139938	Up
mmu-miR-132-3p	1.391856747	0.000172116	Up
mmu-miR-330-5p	1.330735049	0.000188406	Up
mmu-miR-129-2-3p	2.090581397	0.000348231	Up

3.2.4 Validation of RNA sequencing data:

(a) iDC vs iDC (TCM). Based on the previous studies and our RNA seq data, we selected 6 miRNAs from the list of significantly differentially expressed miRNA (based on RNA seq data) which were relevant to dendritic cells in the context of tumor microenvironment. These miRNAs were validated using qRT-PCR analysis. These include miR-155-5p, miR-155-3p, miR-146a-3p, miR-365-2-5p, miR-187-3p, miR-142a-3p. Real time PCR analysis showed that in iDC TCM, expression of miR-155-5p was significantly upregulated 2.27 fold and 1.64 fold in two biological replicates respectively as compared to iDC. In comparison, there was 1.67 fold upregulation in iDC (TCM) by RNA sequencing analysis (fig 30).

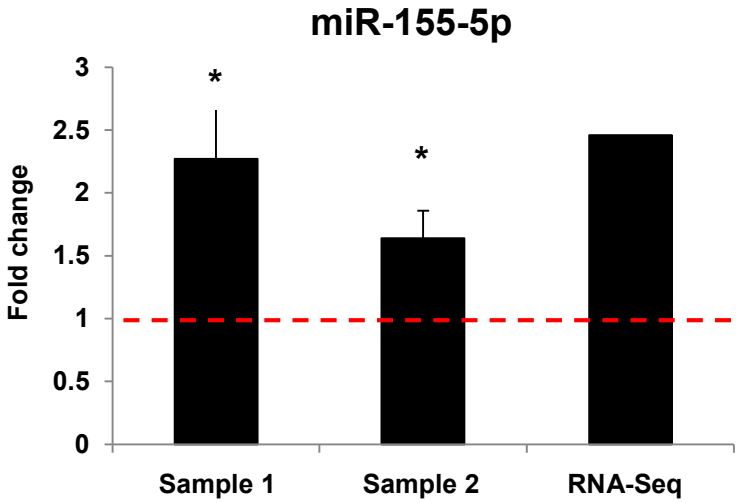


Fig 30: TCM upregulated miR-155-5p in DC. Expression of miR-155-5p was quantified using RT-PCR. Sample 1 and sample 2 represent two biological replicates. Each sample was run in triplicate. Data represent fold change in expression of miR-155-

5p in iDC vs iDC TCM (mean ± s.e.m.) calculated through REST^{TM} software for data analysis (*p<0.05).

Next, expression of miR-155-3p was analyzed using RT-PCR. Fig 31a shows that expression of miR-155-3p was upregulated 8.16 and 13.26 fold in iDC TCM as compared to iDC. In RNA sequencing, expression of miR-155-3p was found to be upregulated 3.58 fold. Similarly, expression of miR-146a-5p was upregulated by 3.01, 6.9 and 2.84 fold in sample 1, sample 2 and RNA sequencing data (Fig 31b).

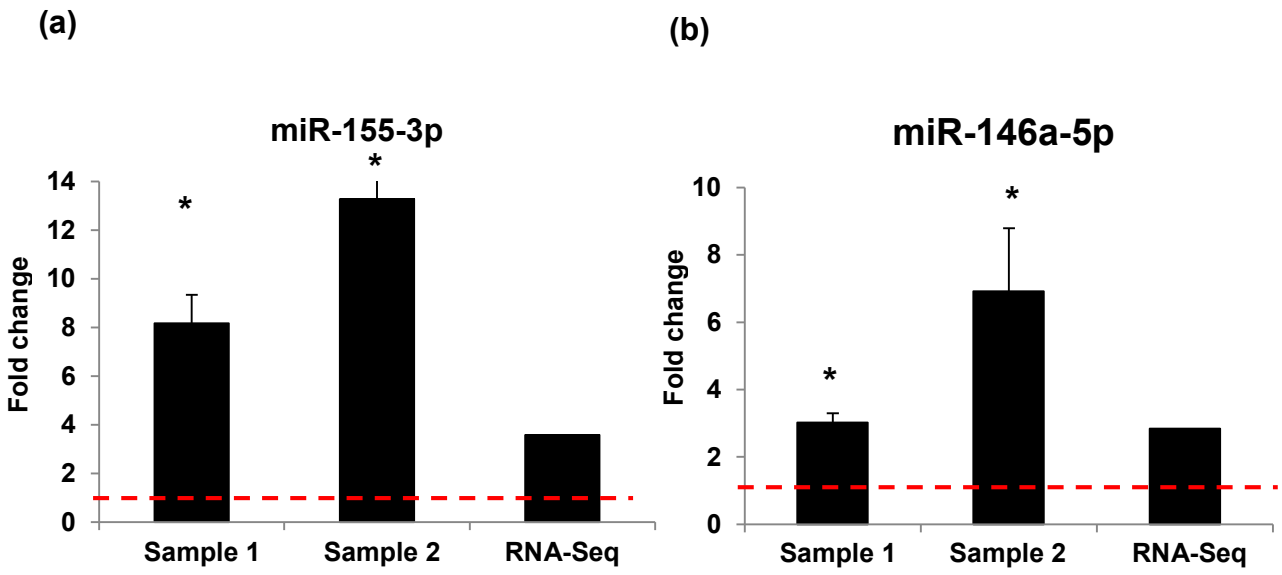


Fig 31: TCM upregulated miR-155-3p and miR-146a-5p in DC. Expression of (a) miR-155-3P (b) miR-146-5p was quantified using RT-PCR. Sample 1 and sample 2 represent two biological replicates. Each sample was run in triplicate. Data represent fold change (mean ± s.e.m.) calculated through REST^{TM} software for data analysis (*p<0.05).

In RNA sequenicng analysis, expression of miR-365-2-5p and miR-187-3p was down regulated by 0.57 fold and 0.65 fold respectevely. Follwing results were validated using RT-PCR analysis and it was observed that expression of miR-365-2-5p was significantly downregulated by 0.532 and 0.457 fold in 2 biological replicates (Fig 32a) while expression of miR-187-3p was downregulated by 0.02 fold and 0.05 fold in two biological replicates of iDC TCM as compared to iDC (Fig 32b)

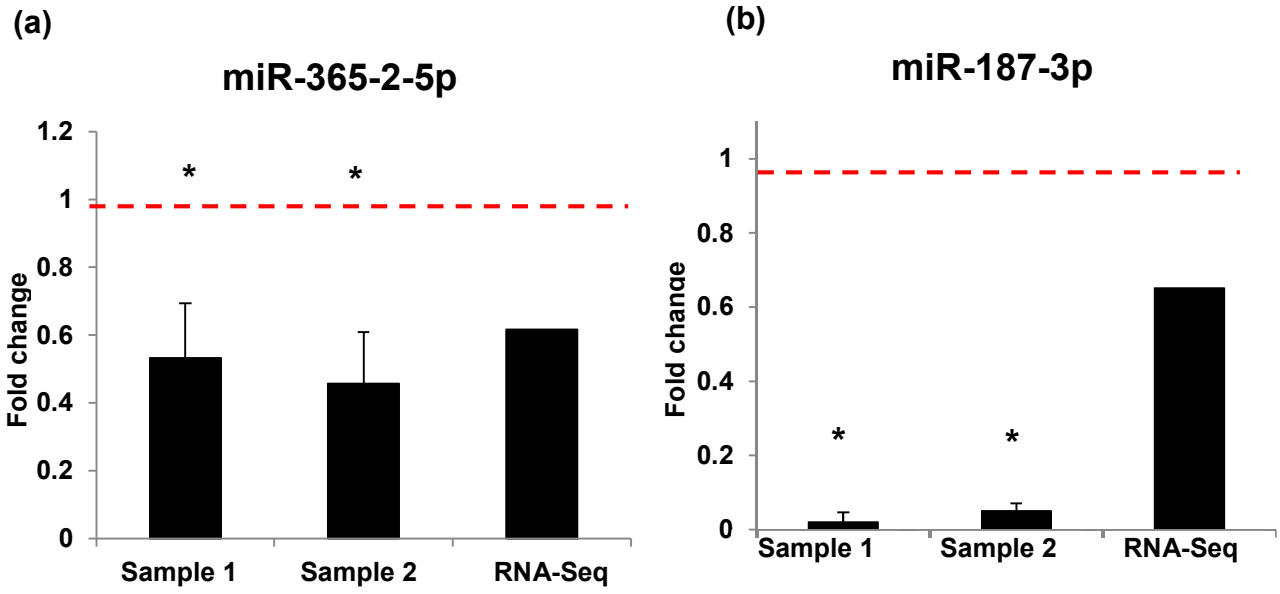


Fig 32: **TCM downregulated miR-365-2-5p and miR-187-3p in DC.** Expression of (a) miR-365-2-5p (b) miR-187-3p was quantified using RT-PCR. Sample 1 and sample 2 represent two biological replicates. Each sample was run in triplicate. Data represent fold change (mean ± s.e.m.) calculated through REST^{TM} software for data analysis (*p<0.05).

Expression of miR-142a-3p was validated using RT-PCR. In RNA sequencing analysis, expression of miR-142a-3p was downregulated by 0.68 fold. However in RT-PCR

analysis, expression of miR-142a-3p was found to be upregulated by 4.68 and 8.54 fold (Fig 33).

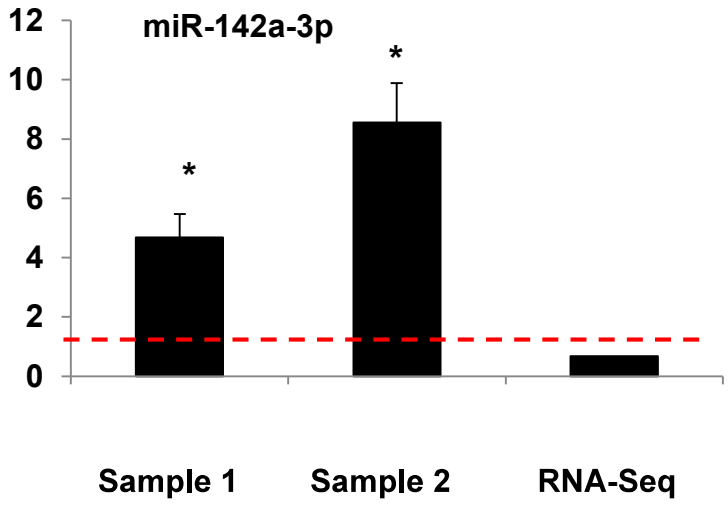


Fig 33: TCM upregulated miR-142a-5p expression in DC. Expression of miR-142a-3p was quantified using RT-PCR. Sample 1 and sample 2 represent two biological replicates. Each sample was run in triplicate. Data represent fold change (mean ± s.e.m.) calculated through REST^{TM} software for data analysis (*p<0.05).

(b) iDC vs mDC : Out of the 6 miRNA whose expression was validated in iDC TCM vs iDC, miR-155-5p, miR-155-3p and miR-365-2-5p was found to be significantly changed in iDC vs mDC as per RNA sequencing data. Thus we further validated the expression of all the six miRNA in iDC and mDC pair. In RT-PCR analysis, it was observed that expression of miR146a-3p, miR-187-3p, miR155-5p, miR-142a-3p were not significantly changed. However the expression of miR-155-3p and miR-365-2-5p was significantly changed in mDC when compared to iDC. Expression of miR-155-3p was upregulated by 3.14 fold in mDC as compared to

iDC, expression miR-365-2-5p was downregulated by 0.75 fold in mDC as compared to iDC (Fig 34a).

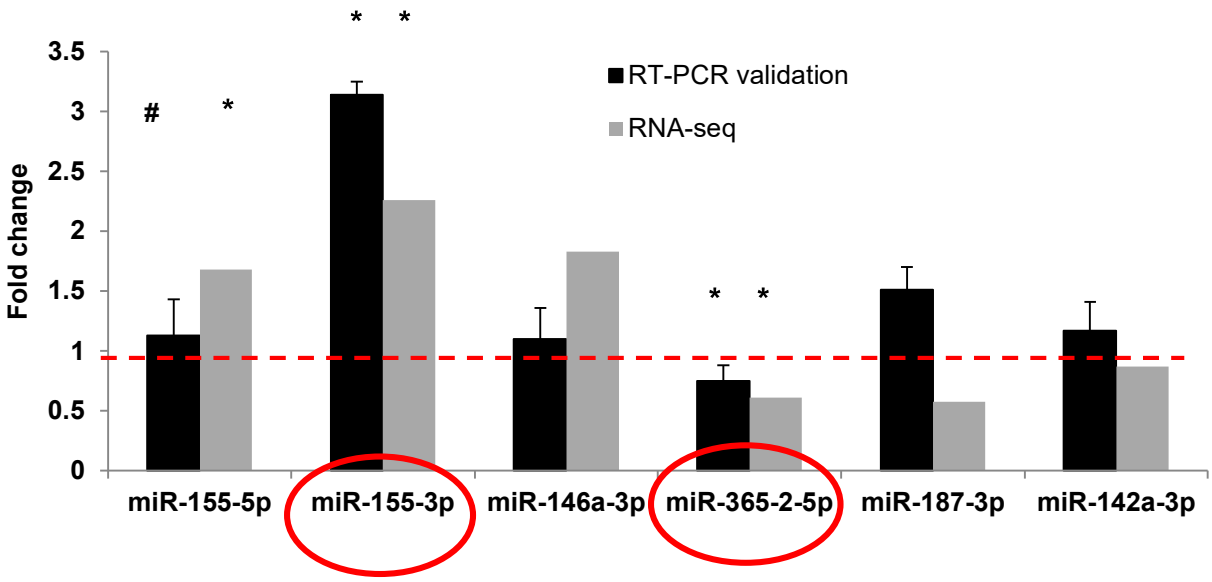


Fig 34: **Differential regulation of miRNAs in DC maturation.** Expression of miR-155-5p, miR155-3p, miR146a-3p, miR-365-3-5p, miR-187-3p, and miR-142a-3p was quantified using RT-PCR. Each sample was run in triplicate. Data represent fold change (mean ± s.e.m.) calculated through REST^{TM} software for data analysis (#:non significant, *p<0.05).

For the 6 miRNAs of interest, a fold change graph was made for both iDC vs iDC (TCM) and iDC vs mDC based on RNA seq data (Fig 35a) and RT-PCR validation data (Fig 35b). The graphs show that three miRNAs (miR-155-5p, miR-155-3p and miR-365-2-5p) had significant differential expression in both iDC vs iDC (TCM) as well as in iDC vs mDC as per RNA seq data.

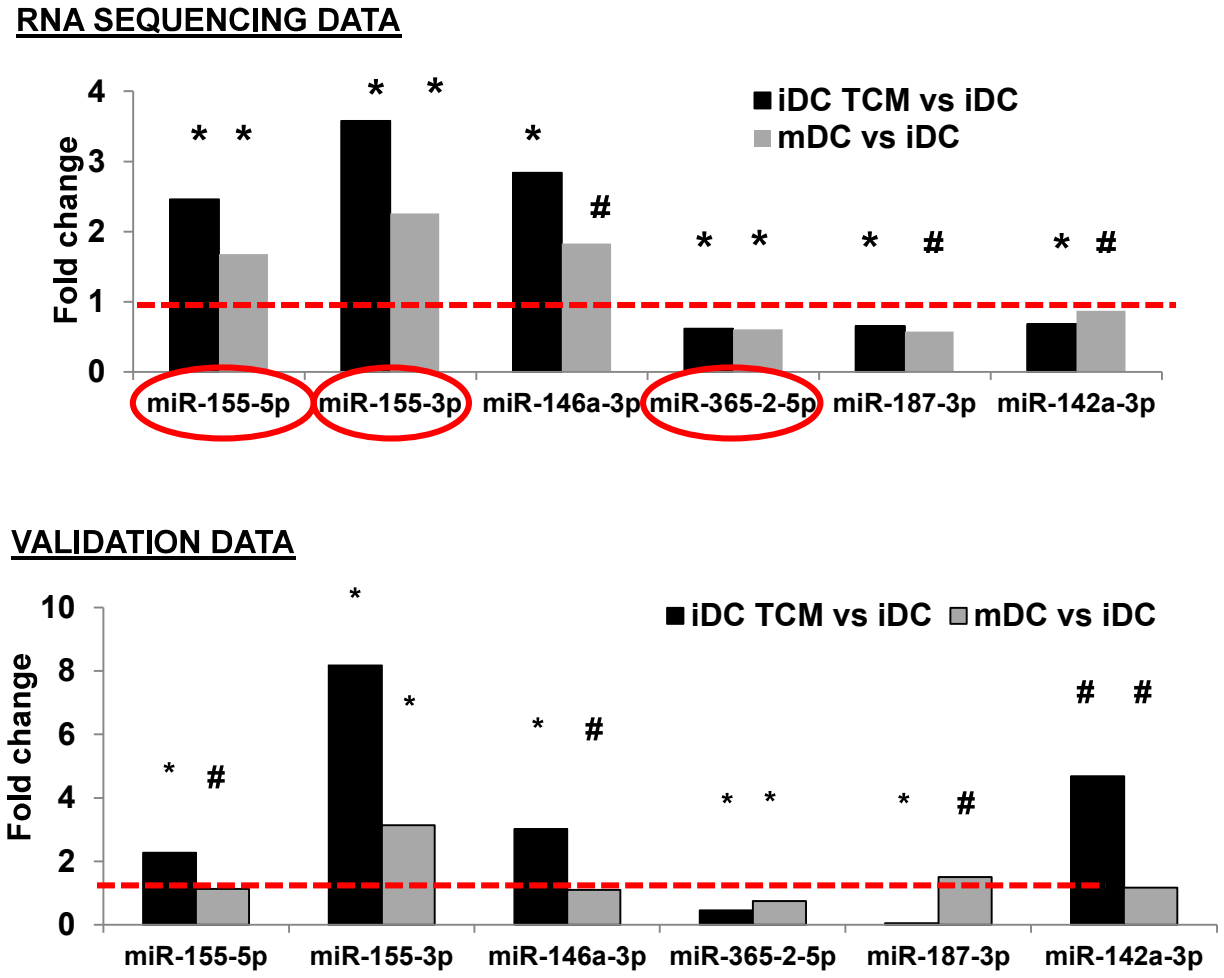


Fig 35: Comparison of selected miRNAs assessed by RT-PCR and RNA seq analysis. Fold change of six miRNAs of interest between iDC vs iDC (TCM) as well as iDC vs mDC based on (a) RNA seq analysis and (b) RT-PCR validation analysis.

3.3 Effect of progenitor cell irradiation on differentiation and maturation of DC.

In order to study the effect of radiation on DC differentiation, bone marrow cells containing DC progenitors were isolated from C57BL/6 mice and irradiated *in vitro*. DC were differentiated from irradiated precursor and analyzed for the expression of phenotypic maturation markers.

3.3.1 Effect of radiation on DC differentiation *in vitro*:

(a) Irradiation of progenitor cells increase BMDC maturation: BMC were isolated and irradiated with different doses of γ-rays (0.5 Gy, 1.0 Gy, 2.0 Gy, 4.0 Gy). Cells were further cultured with GM-CSF and IL-4 to differentiate and mature into DC following standard protocol. Expression of maturation markers was analyzed on immature and mature DC. DC differentiated from irradiated progenitors (0.5 Gy, 1.0 Gy) showed increased expression of CD40, CD80, CD86 and MHC II as compared to DC from unirradiated control progenitors. Expression of CD40 increased from 40% in DC from control progenitors (DC-CP) to 52% in DC from 0.5 Gy irradiated progenitors (DC-0.5 Gy IP) and 54% in DC from 1.0 Gy progenitors (DC-1 Gy IP) (Fig 36a). Although DC from 2.0 Gy irradiated progenitors (DC-2.0 Gy IP) did show increase in CD40 expression to 54%, the increase was not consistent in different experiments. Similarly CD80 expression increased from 38% in DC from DC-CP to 50% in DC-0.5 Gy IP, 50% in DC-1 Gy IP and to 42% in DC-2 Gy IP (Fig 36b). CD86 expression increased from 52% in DC-CP to 70% in DC-0.5 Gy IP, 66% in DC-1 Gy IP and to 55% in DC-2 Gy IP (Fig 36c). MHC II expression was 20%, 30%, 32% and 38% in DC-CP, DC-0.5 Gy IP, DC-1 Gy IP and DC-2 Gy IP respectively (Fig. 36d).

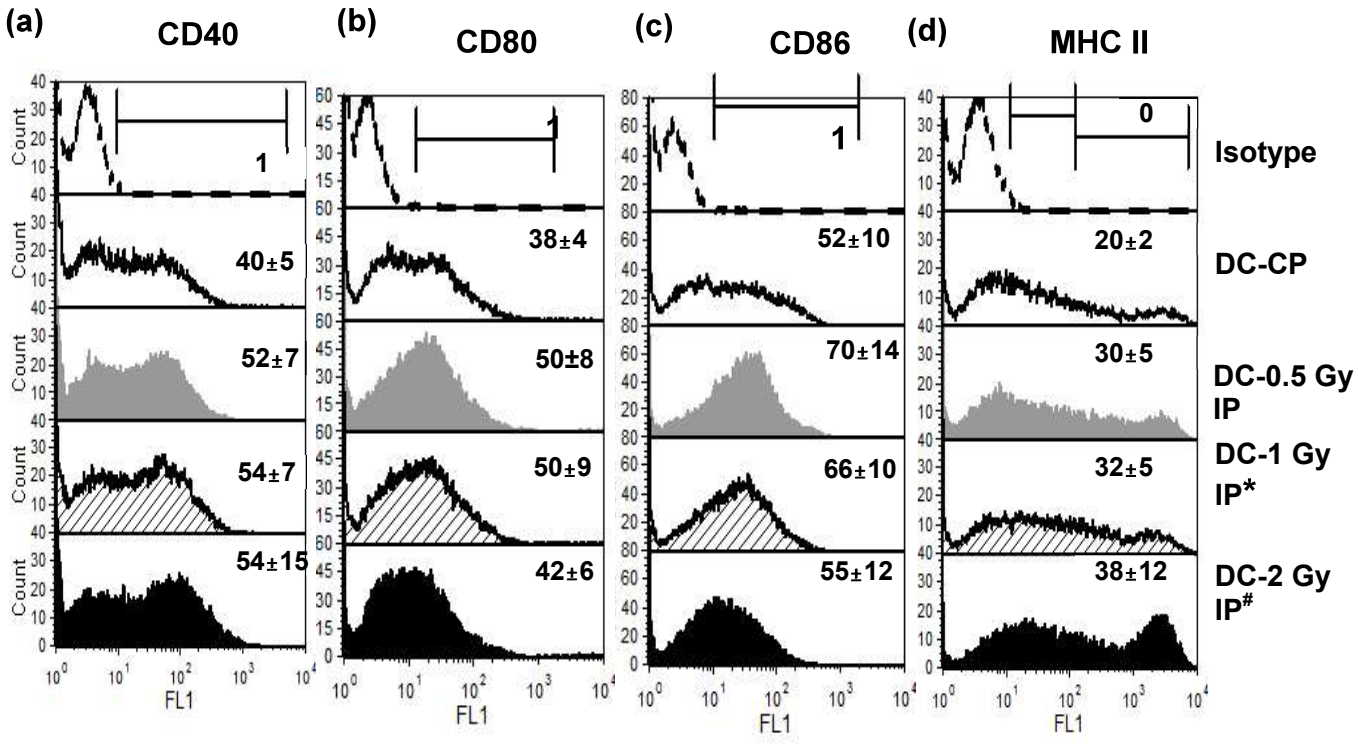


Fig 36: Irradiation of progenitor cells increased phenotypic maturation of BMDC. Bone marrow cells were isolated from C57BL/6 mice and exposed to different doses of γ irradiation (0.5 Gy, 1.0 Gy and 2.0 Gy). BMDC were generated and expression of (a) CD40 (b) CD80 (c) CD86 and (d) MHC II was analyzed using flow cytometry. Histogram shown here is from one representative experiment. Figures in histograms are mean of percentage positive cells from three independent experiments (*$p<0.05$ between DC-CP vs DC-0.5 Gy IP and DC-1.0 Gy IP; **#:** non-significant between DC-CP vs DC-2 Gy-IP).

(b) BMDC from irradiated progenitors showed increased IL-12 and TNF-α secretion: BMDC differentiated from unirradiated control and irradiated progenitors were analyzed for the presence of characteristic DC cytokines IL-12 and TNFα using ELISA. There was significant increase in secretion of both IL-12 and TNFα in DC differentiated from 0.5 Gy and 1.0 Gy progenitors. In DC from

2.0 Gy irradiated progenitors, though there was an increase compared to unirradiated control, it was much less than 0.5 Gy and 1.0 Gy BMDC (Fig 37).

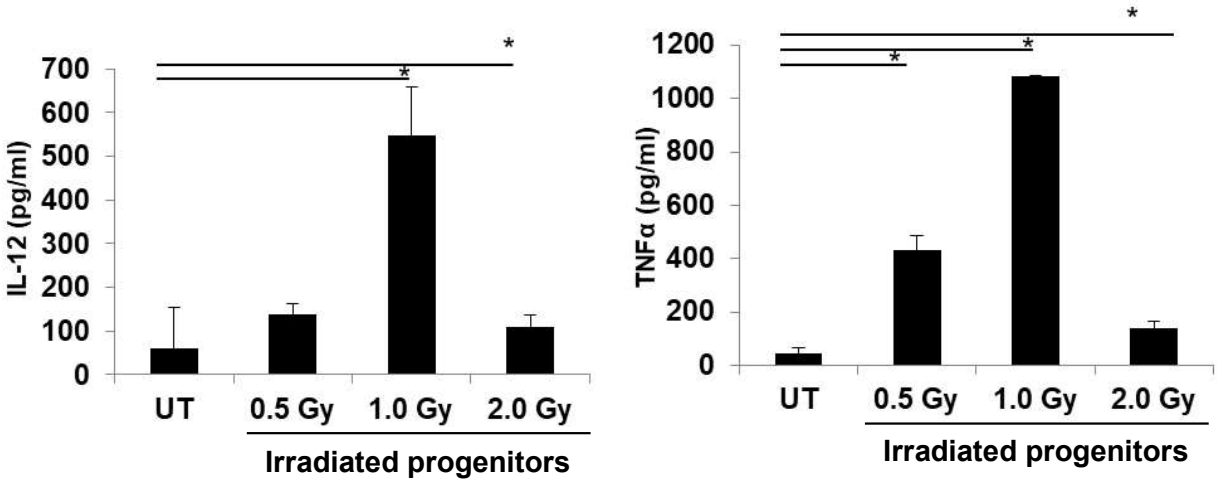


Fig 37: BMDC from irradiated progenitors showed increased level of IL-12 and TNFα. Culture supernatants were evaluated for presence of (a) IL-12 and (b) TNFα cytokine using ELISA. Data shown is mean±s.e.m. from a representative experiment in triplicate (*p<0.05).

(c) BMDC from irradiated progenitors showed no change in phagocytosis capacity: Phagocytic ability of BMDC generated from irradiated progenitor cells was assessed using *E.coli* bioparticle. Cells were incubated with FITC tagged *E.coli* bioparticle at 4^0C and 37^0C and percentage phagocytosis was calculated by subtracting the value of percentage positive cells at 4^0C from % positive cells at 37^0C. We did not observe any significant change in phagocytosis ability of DC-CP, DC-0.5 Gy IP and DC-1.0 Gy IP (Fig 38).

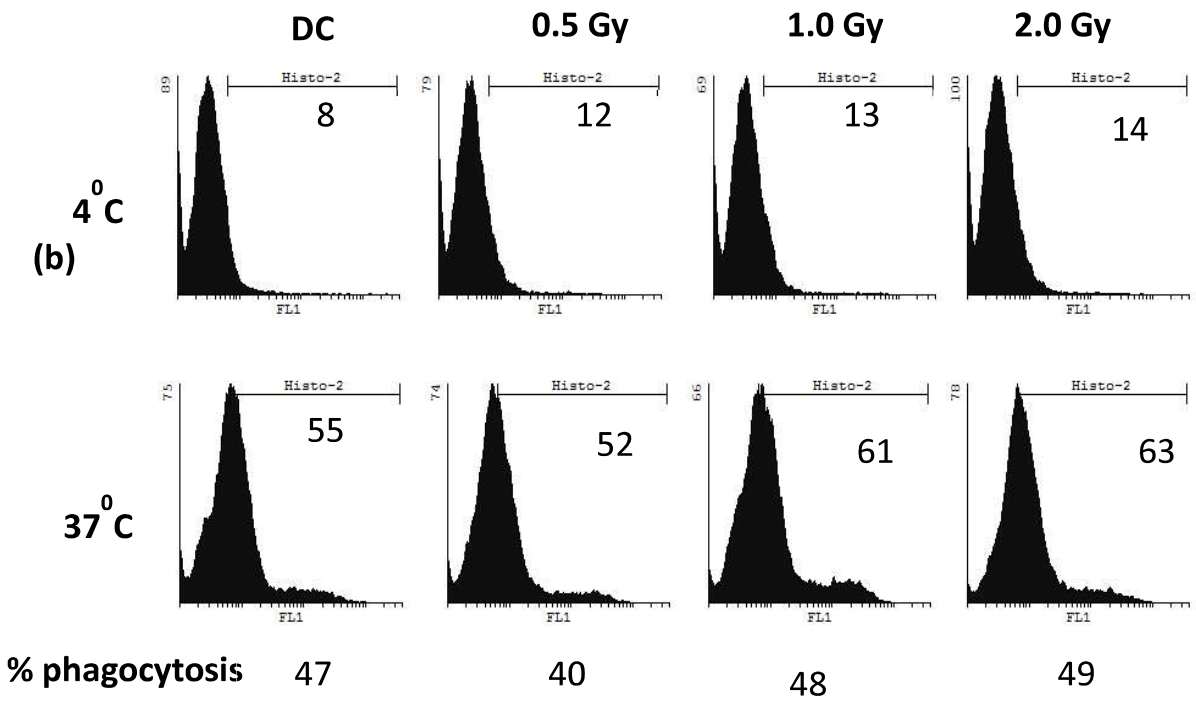


Fig 38: BMDC from irradiated precursors did not alter phagocytosis capacity of DC. (a) BMDC were cultured with *E.coli* bioparticles for 1 h at 37^0C. (b) Cells incubated at 4^0C served as control. Percentage phagocytosis was measured as difference between the percentage positive cells at 37^0C and 4^0C. Data shown here is one representative experiment from two independent experiments.

(d) Irradiation of progenitor cells increased the cross presentation ability of BMDC: Cross presentation ability of BMDC was analyzed by two methods. Different DC sets were pulsed with SIINFEKL peptide and D1.16 antibody was used to quantify the presence of SIINFEKL peptide in context of H-2^b MHC I molecules. It was observed that percentage positive cells for D1.16 increased from 8% in DC-CP to 20% in DC-0.5 Gy IP and 30% in DC-1.0 Gy IP (Fig. 39a) In the other method, SIINFEKL peptide pulsed DC were incubated with B3Z T

cell hybridoma expressing β-galactosidase and CPRG substrate was used to measure the activation of B3Z cells as discussed earlier. It was observed that there was significant increase in activity of β-galactosidase (Fig 39b) in both DC-0.5 Gy IP and DC-1.0 Gy IP. Both these experiments indicated that irradiation of progenitor cells increased the cross presentation ability of BMDC.

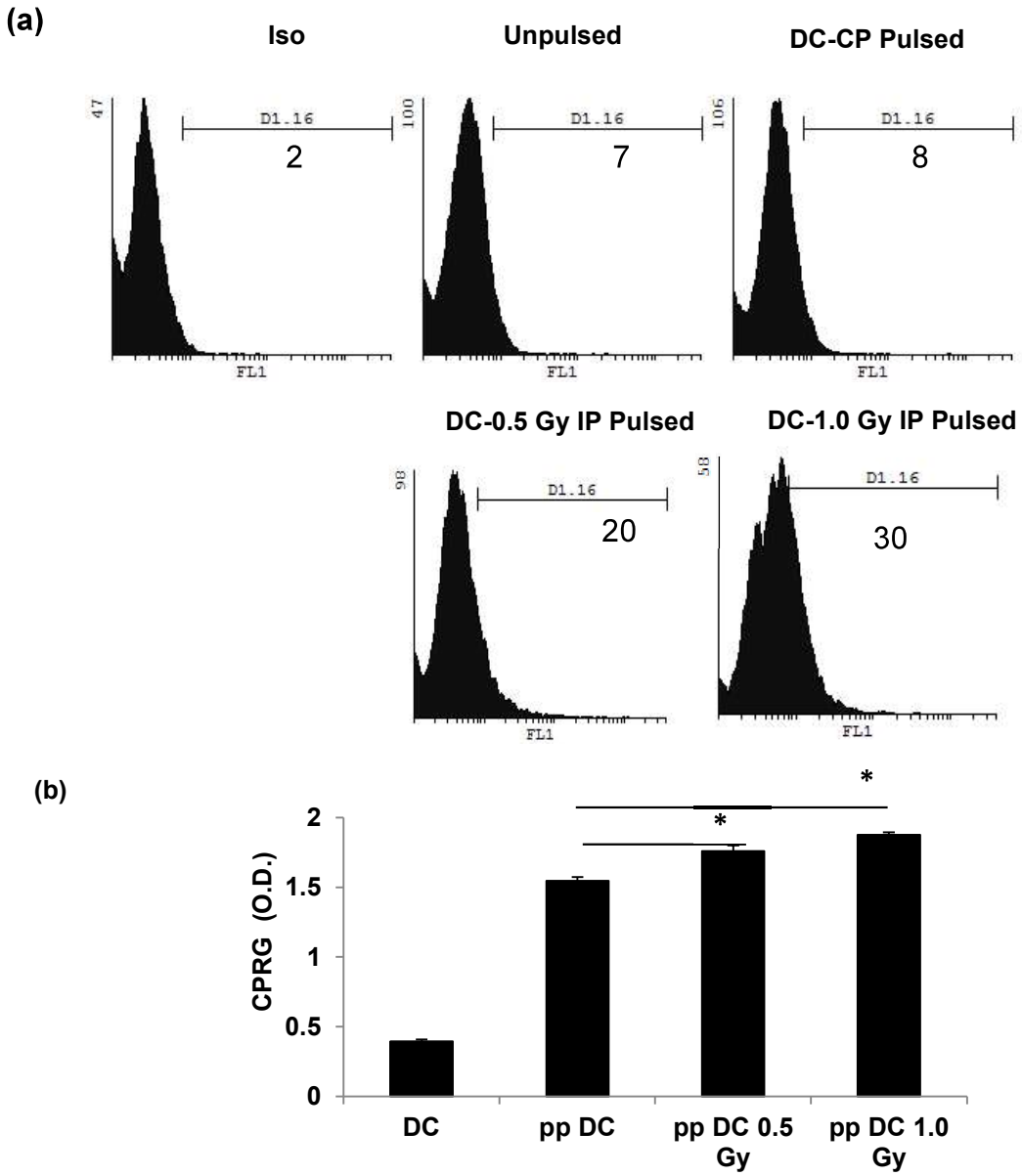


Fig 39: BMDC from irradiated progenitors showed upregulation of cross

presentation activity. (a) SIINFEKL peptide pulsed BMDC were labelled with D1.16 antibody and evaluated using flow cytometry. Value in histogram is percentage positive cells. Data shown here is representative from two independent experiments. (b) Activation of B3Z T cells was evaluated by quantifying the expression of galactosidase in B3Z cells co-cultured with SIINFEKL peptide pulsed BMDC by using CPRG as substrate. CPRG absorbance was measured at 570 nm. Data are mean ± s.e.m. of triplicates (*$p<0.05$).

3.3.2 Role of apoptosis in upregulation of DC immunogenicity:

(a) Apoptotic profile of BMC in presence and absence of GM-CSF: Bone marrow cells were irradiated as described earlier and cultured for different time points in presence or absence of GM-CSF. Cells were harvested and stained with PI and cell cycle analysis was carried out using flow cytometry. There was a dose dependent increase in percentage apoptotic cells in presence and absence of GM-CSF at 24 h. This increase in apoptotic cells was seen at all-time points in cells cultured without GM-CSF. However, when GM-CSF was present there was dose dependent increase in apoptotic cells only 24 h after radiation and no increase was observed at 48 h and 72 h (Fig 40).

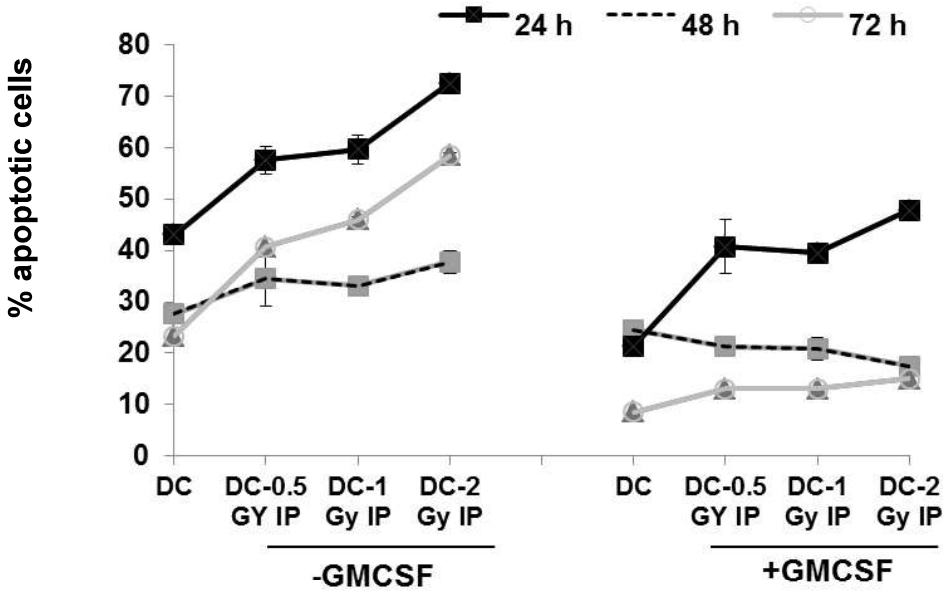


Fig 40. **Apoptotic profile of irradiated BMC with GM-CSF.** Bone marrow cells ($1X10^6$) were irradiated with different doses of ionizing radiation (0.5 Gy, 1.0 Gy, 2.0 Gy) and further cultured with and without GM-CSF. Cells were fixed and stained with PI at 24 h, 48h and 72 h. Cell cycle analysis was carried out by flow cytometry. Cells with less than G1 DNA content were enumerated as apoptotic cells. Values are mean ± s.e.m. of three replicates. Comparison were made between –GM-CSF and +GM-CSF groups in corresponding time points (*$p<0.05$).

(c) Role of apoptotic cells in irradiation induced increase in DC immunogenicity:

Since there was an increase in apoptotic cells in 24 h after irradiation in presence of GM-CSF, and no increase in later time points, we explored the role of apoptotic cells in irradiation induced DC immunogenicity. Irradiated DC precursor cells were cultured for 24 h in DC differentiation media. Cells were harvested and apoptotic/dead cells were removed from live cells using Ficoll-Histopaque density gradient separation. Viable cells were carefully removed from the top of ficoll and

cells were counted and further cultured in DC differentiation media containing GM-CSF and IL-4. Following treatments, there were 4 sets of DC culture: DC-0.5 Gy IP, DC from ficoll separated irradiated progenitors (DC-0.5 Gy IP+ ficoll) and DC from only ficoll separation (DC-CP ficoll) along with DC-CP. Expression of maturation markers were measured in all sets after differentiation. Expression of CD40 increased from 50% in DC-CP to 64% in DC-0.5 Gy IP, however in DC-0.5 Gy IP + ficoll, expression of CD40 was 49%, which was similar to DC-CP and DC-CP ficoll (47%) (Fig 41a). Expression of CD80 was 38%, 54% 37%, 40% in DC-CP, DC-0.5 Gy IP, DC-0.5 Gy IP + ficoll and DC-CP ficoll respectively (Fig 41b). Similarly expression of MHCII was 36%, 52%, 40%, 42% in in DC-CP, DC-0.5 Gy IP, DC-0.5 Gy IP + ficoll and DC-CP ficoll respectively (Fig 41c). This experiment showed that if apoptotic cells were removed from irradiated progenitors then increase in immunogenicity of DC observed after irradiation of BMC was not seen, indicating the possible role of apoptotic cells in increase in DC immunogenicity.

(c) Role of HMGB1, Hsp70 and calreticulin in increase in DC immunogenicity:

As the earlier experiments suggested the possible role of apoptotic cells in increase of DC immunogenicity in DC IP, we further explored the factors secreted by apoptotic cells which has been studied and shown to play a role in increase in DC immunogenicity. It was reported that HMGB1 secreted by irradiated cancer cells activated the dendritic cells in TLR dependent pathway (*164*). Similarly role of Hsp70 and calreticulin secreted by apoptotic cancer cell in activating DC has been reported too (*211*).

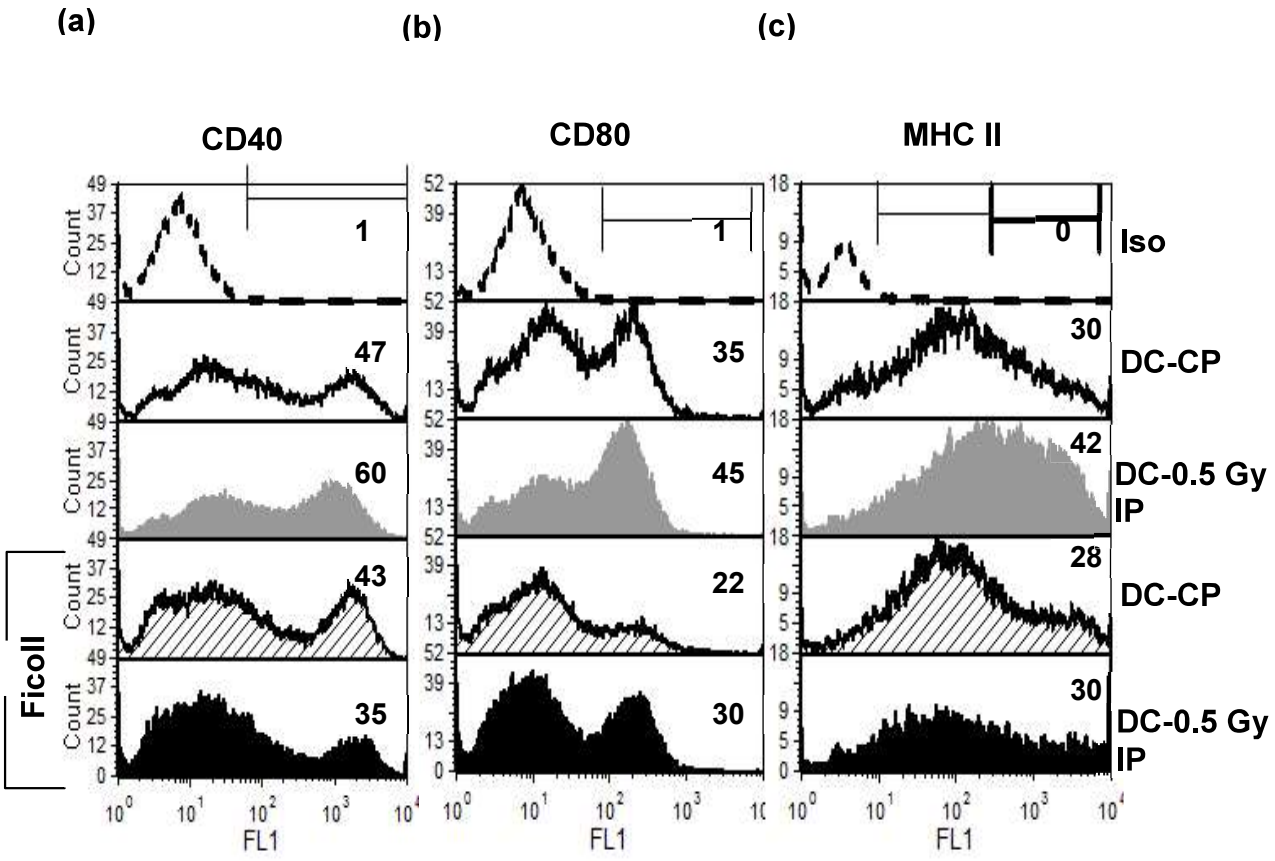


Fig 41: Removal of apoptotic cells abrogated increase in radiation induced DC maturation. Viable cells were separated from overnight culture of irradiated bone marrow cells using ficoll. Cells were further cultured for DC differentiation. Expression of (a) CD40 (b) CD80 (c) MHCII was analyzed by flow cytometry. Numbers given in each histogram represents percentage positive cells in gated population. Representative data from two independent experiments is shown.

In this context we analyzed the expression of HMGB1, Hsp70 and calreticulin in bone marrow cells 2 days after irradiation using flow cytometry. There was no change in expression of HMGB1 between groups. It was 37%, 34% and 36% in DC-CP, DC-0.5 Gy IP and DC-1.0 Gy IP respectively (Fig 42a). Similarly the expression of calreticulin

was 25%, 28% and 29% in in DC-CP, DC-0.5 Gy IP and DC-1.0 Gy IP respectively (Fig 42b). When expression of Hsp70 was quantified, the expression of $Hsp70^{high}$ was 62%, 45%, 36% in DC-CP, DC-0.5 Gy IP and DC-1.0 Gy IP respectively (Fig 42c). There was a significant decrease in Hsp70 in irradiated bone marrow cells. The significance of Hsp70 in increase in DC immunogenicity needs to be explored further.

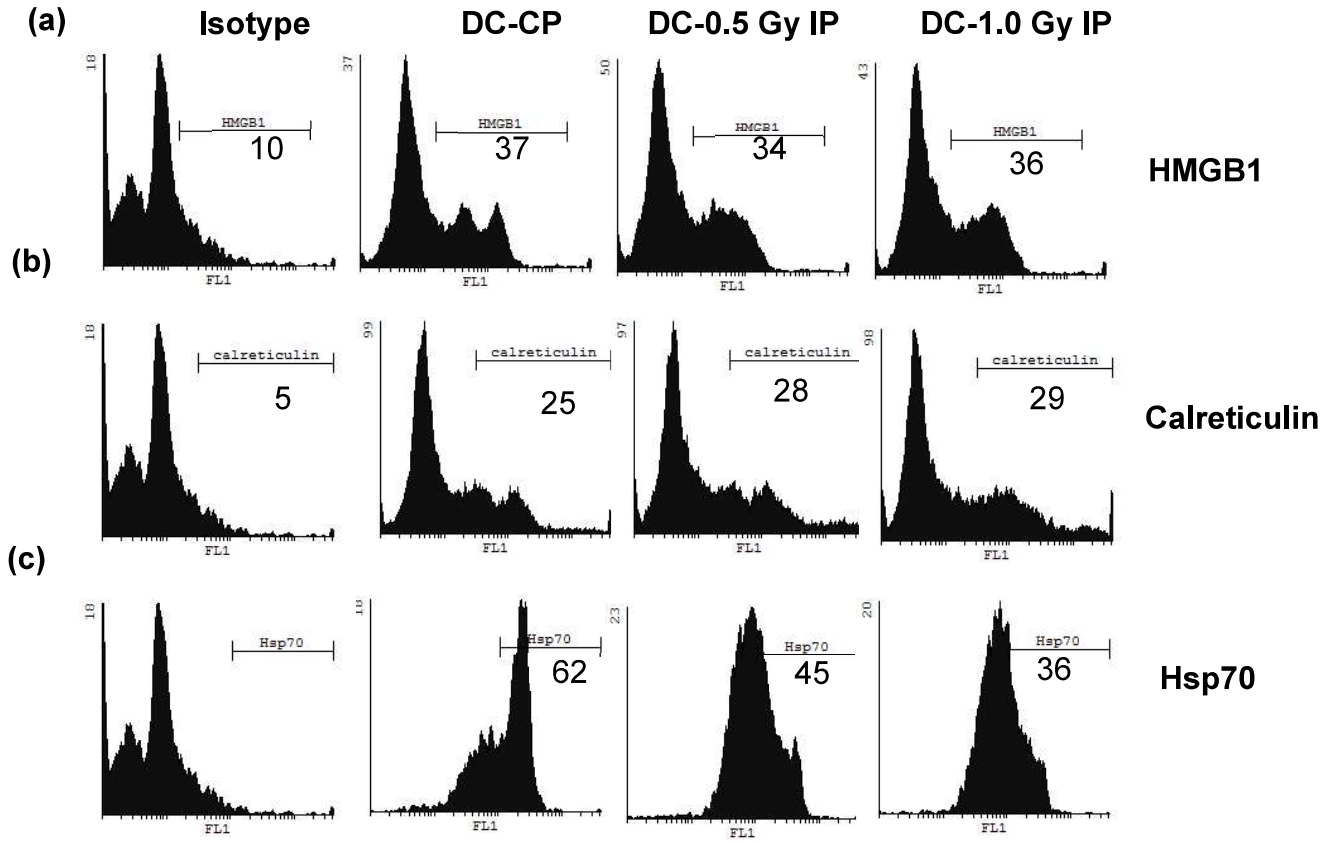


Fig 42: Role of HMGB1, calreticulin and Hsp70 in irradiation induced increase in DC maturation. Expression of (a) HMGB1 (b) calreticulin (c) Hsp70 was quantified using flow cytometry. Number in histogram represents percentage positive cells. Data represented here is a representative from two independent experiments.

(d) Radiation induced STAT3 phosphorylation in DC. *In vitro* DC differentiation by GM-CSF and IL-4 involves different components of STAT signaling (*212*). STAT3 is known to regulate cDC lineage while STAT5 is involved in pDC lineage. In order to study the lineage preference of DC differentiated from irradiated progenitors, expression of phospho-STAT3 and STAT5 were analyzed in DC-IP and DC-CP. Basal level of STAT3 expression was decreased in DC-0.5 Gy IP as compared to DC-CP while basal level of phospho-STAT5 was increased in DC-0.5 Gy IP as compared to DC-CP (Fig 43a-b). Similar trend was observed in western blot analysis (Fig 43c).

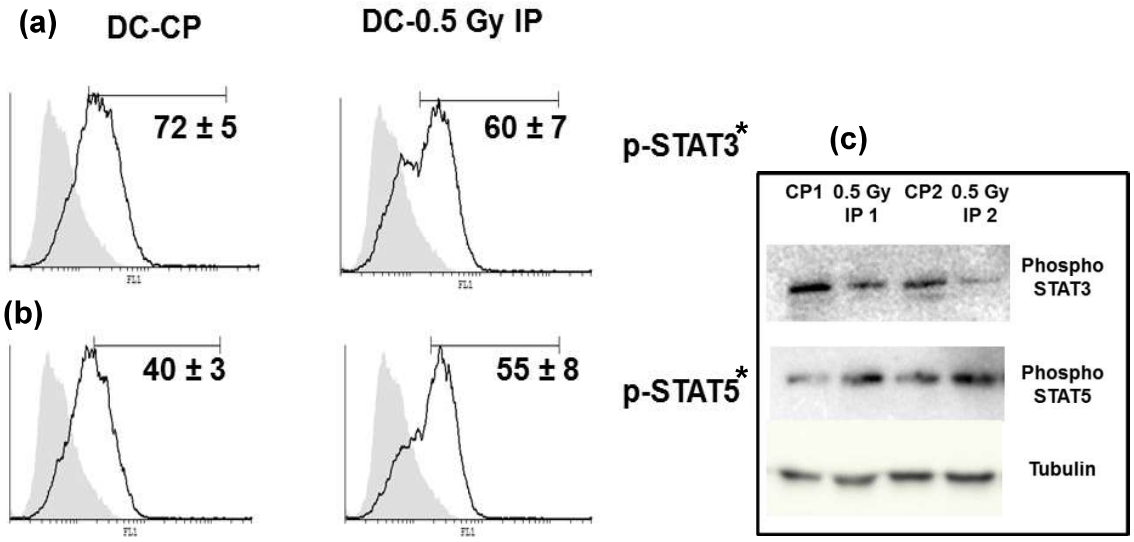


Fig 43: Irradiation of BMC progenitors modulate STAT signaling. Expression of (a) p-STAT3 (b) p-STAT5 was quantified using flow cytometry. Number in histogram represents percentage positive cells. Data represented here is a representative from three independent experiments (Comparison between DC-CP vs DC-0.5 Gy IP,

*p<0.05). (c) Western blot analysis of DC-CP and DC-0.5 Gy IP of phospho-STAT3 and phospho-STAT5 from biological replicates.

3.3.3 Effect of radiation on DC differentiation *in vivo*:

(a) Effect of whole body irradiation on splenic DC: In order to study the effect of radiation on *in vivo* differentiated splenic DC, mice were exposed to whole body irradiation of 0.5 Gy and 1.0 Gy along with unirradiated control and 24 h later, mice were sacrificed and splenic DC were isolated using magnetic microbeads and expression of maturation markers were analyzed. There was no significant change in expression of CD40 and MHC II. Expression of CD40 was 52% in control sDC from unirradiated mice as compared to 46% in sDC from 0.5 Gy WBI and 50% in 1.0 Gy WBI sDC (Fig 44a). Similarly expression of MHCII was 58%, 62% and 63% in sDC: control, sDC: 0.5 Gy and sDC: 1.0 Gy respectively (Fig 44 b). These results suggested that increase in immunogenicity of DC as observed in irradiated progenitors was not seen in splenic DC which were terminally differentiated.

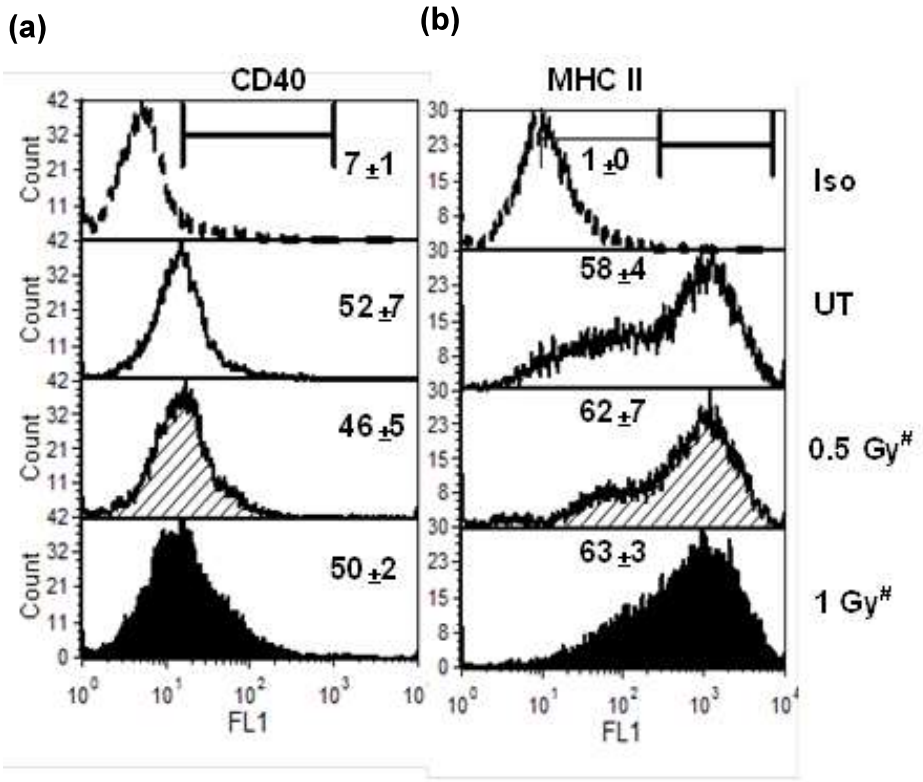


Fig 44. WBI of mice did not alter the maturation status of splenic DC. Mice were exposed to WBI of 0.5 Gy or 1.0 Gy along with control. Splenic DC were isolated using $CD11c^{+}$ magnetic microbeads and expression of (a) CD40 and (b) MHCII was analyzed using flow cytometry. Histograms are from a representative experiment and the values in the histogram are mean ± s.e.m. of percentage positive cells from three experiments (#: not significant).

(b) Effect of whole body irradiation (WBI) on differentiation of murine BMDC: C57BL/6 mice were exposed to WBI of 0.5, 1 and 2 Gy to understand the effect of *in vivo* irradiation on differentiation of bone marrow derived DC. Mice were sacrificed 24 h after WBI, and bone marrow cells were differentiated with GM-CSF for generation of DC following standard protocol as well as assessed for cells undergoing apoptosis. With increasing doses of radiation, there was an increase in

apoptosis of bone marrow cells as compared to 5% apoptosis in unirradiated control BMC, apoptosis in 0.5 Gy, 1.0 Gy and 2.0 Gy WBI BMC were 9%, 12% and 20% respectively (Fig 45a).

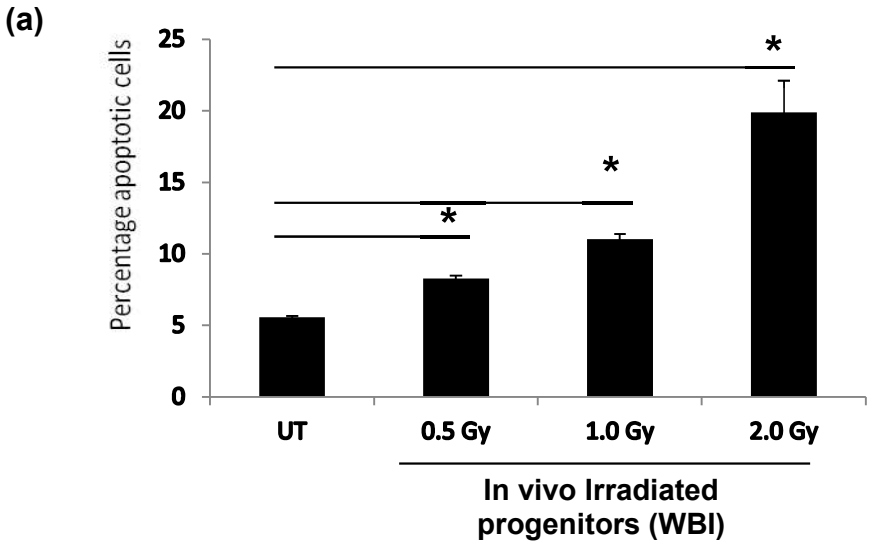


Fig 45: Effect of WBI on differentiation of BMDC. Whole body irradiation induced apoptosis in bone marrow cells. Mice were exposed to WBI of 0.5 Gy, 1.0 Gy and 2.0 Gy. After 24 h, bone marrow cells were isolated and (a) apoptosis was evaluated in BMC using PI staining and flow cytometry. Cells having sub G1 DNA content were enumerated as apoptotic cells. Data are mean ± s.e.m. of triplicates.* $p<0.05$.

When expression of maturation markers was evaluated on BMDC differentiated from same set of WBI mice, it was observed that expression of CD40 increased from 35% in unirradiated control to 44% in 0.5 Gy WBI, 48% in 1.0 Gy and 63% in 2.0 Gy WBI DC (Fig 45b). Similarly expression of $MHCII^{High}$ was 15%, 21%, 22% and 32% in unirradiated control, 0.5 Gy WBI, 1.0 Gy WBI and 2.0 Gy WBI DC (Fig 45c). The increase in DC immunogenicity in BMDC from WBI mice, followed a slightly different

pattern as compared to *in vitro* irradiated BMC, since here the effect was dose dependent and was seen in 2.0 Gy WBI also.

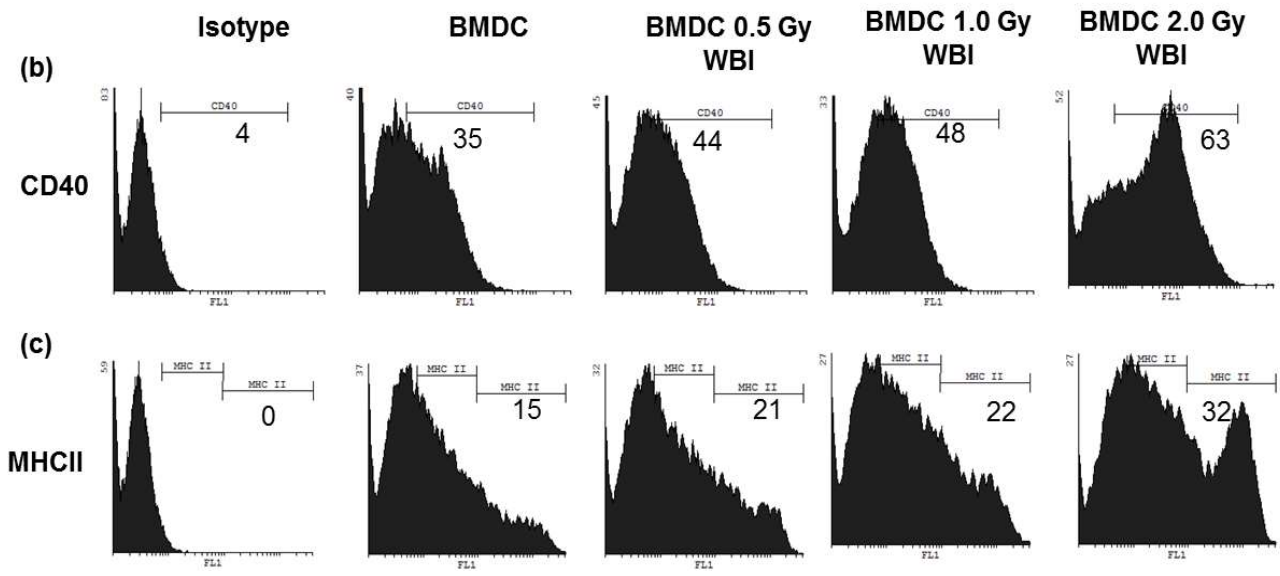


Figure 45: Effect of WBI on differentiation of BMDC: Bone marrow cells from WBI mice (0.5 Gy, 1.0 Gy, 2.0 Gy) were cultured for DC differentiation. Expression analysis of (b) CD40 (c) MHCII on BMDC was evaluated by flow cytometry. Numbers in each histogram represent the percentage of positive cells in each group. Data shown here is a representative from two independent experiments.

(c) Effect of radio-adaptive response on differentiation of murine BMDC

In order to confirm the role of apoptotic cells in increased maturation of DC, mice were first exposed to WBI of a low priming dose followed by a WBI of a high challenge dose. Mice were exposed to priming dose alone (10 cGy), challenge dose alone (2 Gy) or priming and challenge dose 10 cGy + 2 Gy) along with unirradiated control. The time interval between the priming and challenge dose was 4 h. Twenty four hours after challenge dose, mice were sacrificed and bone

marrow cells were isolated. Further BMC were evaluated for induction of apoptosis (cell cycle analysis) as well as incubated with DC differentiation media for generation of BMDC. Expression of CD40/80/86 and MHCII were analysed thereafter.

There was no significant difference in pre G1 population between control and BMC derived from 10 cGy exposed mice. More than two fold increase was observed in pre-G1 population in BMC isolated from mice exposed to 2 Gy, however there was significant decrease ($p < 0.05$) in apoptotic BMC in WBI of 10 cGy + 2 Gy (Fig 46a).

Same set of irradiated BMC were further cultured and differentiated into BMDC and expression of CD40/80/MHCII were analyzed through flow cytometry. It was observed that expression of CD40 was 21% in UT DC and and 22% in 10 cGy WBI DC. In 2 Gy WBI DC CD40 expression was increased to 35%, However DC generated from mice exposed to 10 cGy followed by 2 Gy showed a decrease in the expression of CD40 (24%) as compared to 2 Gy alone (Fig 46b). Similarly the expression of CD80 was also increased to 27% from 21% with 2 Gy WBI compared to unirradiated DC and increase was abrogated to 18% in DC generated from mice exposed to 10 cGy followed by 2 Gy (Fig 46c). MHC II too showed similar pattern where its expression was 18%, 19%, 30%, 22% in DC, DC (10 cGy), DC (2 Gy) and DC (10 cGy + 2Gy) respectively.

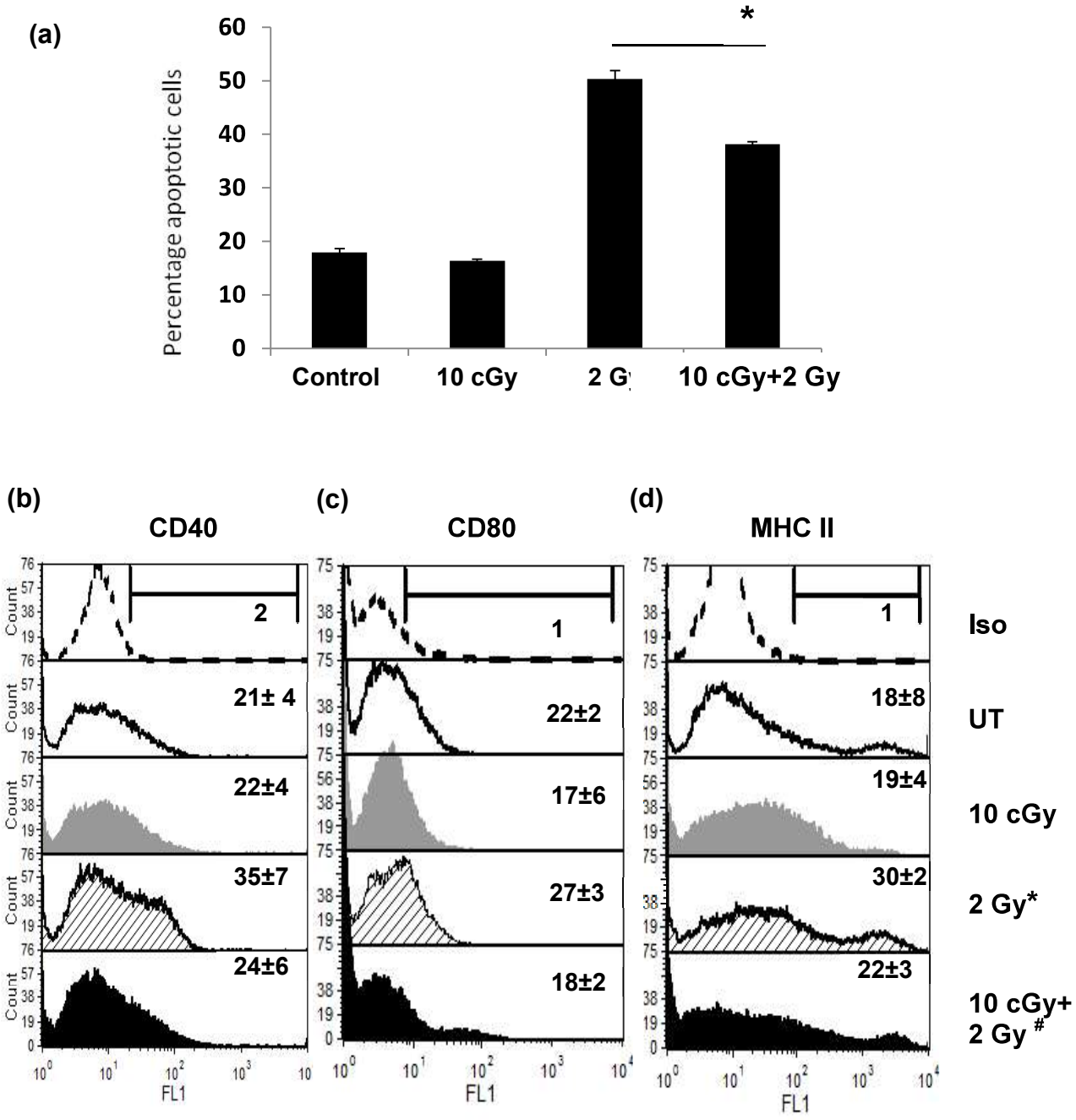


Fig 46. Adaptive response of bone marrow cells confirmed role of apoptotic cells in radiation induced increase in DC maturation. Mice were exposed to WBI of 10 cGy alone, 2 Gy alone and 10 cGy followed by 2 Gy along with control group. (a) After 24 h of WBI, bone marrow cells were isolated and labelled with PI (50 μg/ml) for

analysis of apoptotic cells. Histogram represents the pre-G1 population of cells as percentage apoptotic cells. Expression of (b) CD40 (c) CD80 (d) MHCII was analyzed by flow cytometry. Numbers given in each histogram represents percentage positive cells in gated population (*p<0.05 between UT vs 2 Gy; #: not significant between UT vs 10 cGy+2 Gy).

3.3.4 Transcriptional regulation of DC differentiated from irradiated precursor:

The mRNA profile of lineage specific transcriptional regulators (id2, Bcl6, Zbt46, Batf3 and E2-2) was assessed in BMDC from irradiated and un-irradiated precursors. Consistent increase in relative expression of Zbtb46 transcription factor in BMDC from 0.5 Gy, 1.0 Gy and 2.0 Gy irradiated precursor was observed. BMDC from 0.5 Gy and 1.0 Gy irradiated precursor also showed increase in expression of Id2 transcription factor which was not observed in BMDC from 2.0 Gy irradiated precursor (Fig 47).

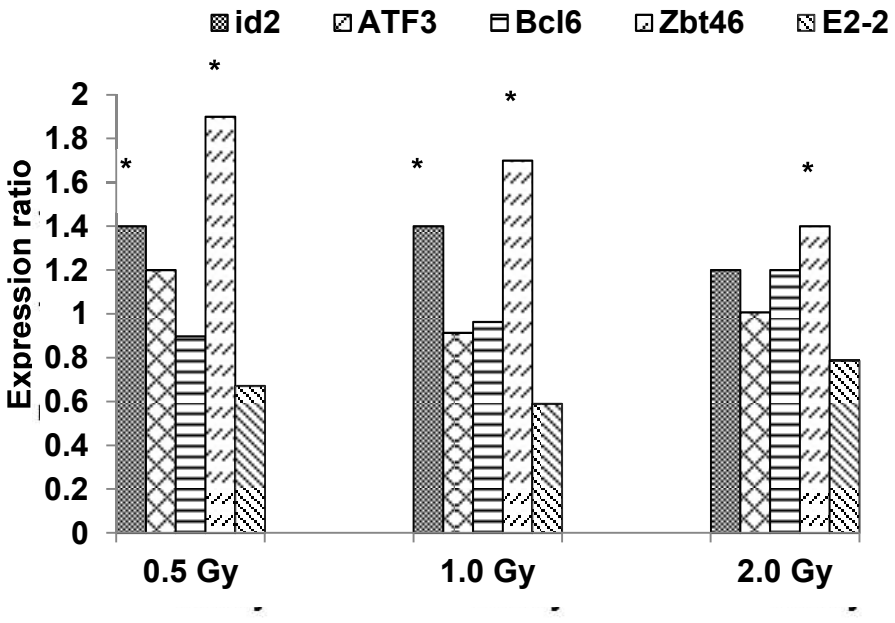


Fig 47. Expression profile of myeloid lineage specific transcription factors in BMDC from irradiated progenitors. Bone marrow cells were isolated and exposed to

0.5 Gy, 1.0 Gy and 2.0 Gy. BMDC were generated following standard protocol. $CD11c^+$ iDC were magnetically sorted and total RNA were extracted and cDNA was synthesized. Quantitative RT-PCR (qRT-PCR) was performed using primers specific for target genes. Data shown here are from a representative experiment with triplicate sample. Cp value was calculated using Roche light cycler 480 analysis software, Expression analysis and statistical significance was calculated using REST software from Qiagen.*$p<0.05$.

3.3.5 TCM did not induce suppression of maturation markers in DC generated from irradiated progenitors: Chapter 3.1 described that the EL4 derived TCM suppressed phenotypic and functional differentiation of DC. This chapter so far described that DC differentiated from irradiated progenitor cells had higher phenotypic maturation as compared to DC-CP. In this context, it was pertinent to explore whether TCM induced suppression was observed in DC derived from irradiated progenitors.

(a) Phenotypic status of DC IP with TCM. BMC were isolated and irradiated *in vitro* with 1 Gy of radiation and further cultured with DC differentiation media for generation of BMDC. In one set the irradiated BMC culture was also supplemented with 20% TCM from EL4 cells. After 8 days, cells were analyzed for maturation status using expression of CD40, CD80 and MHCII. It was observed that expression of CD40, 80 and MHCII were down regulated from 32%, 22% and 66% respectively in control DC to 15%, 15% and 45% respectively in DC (TCM) (Fig 48a-c). While in DC (1 Gy) CD40/80/MHCII expression was 45%, 40% and 76% respectively. In DC-1 Gy IP (TCM) expression was 35%, 35%, and 58%. Although in DC-1 Gy IP (TCM), expression

level was not as high as DC-1 Gy IP but it was significantly higher compared to DC (TCM). There was no significant difference/suppression between DC control and DC-1Gy IP TCM. These results suggested that TCM induced immunosuppression was not observed in DC derived from irradiated progenitors.

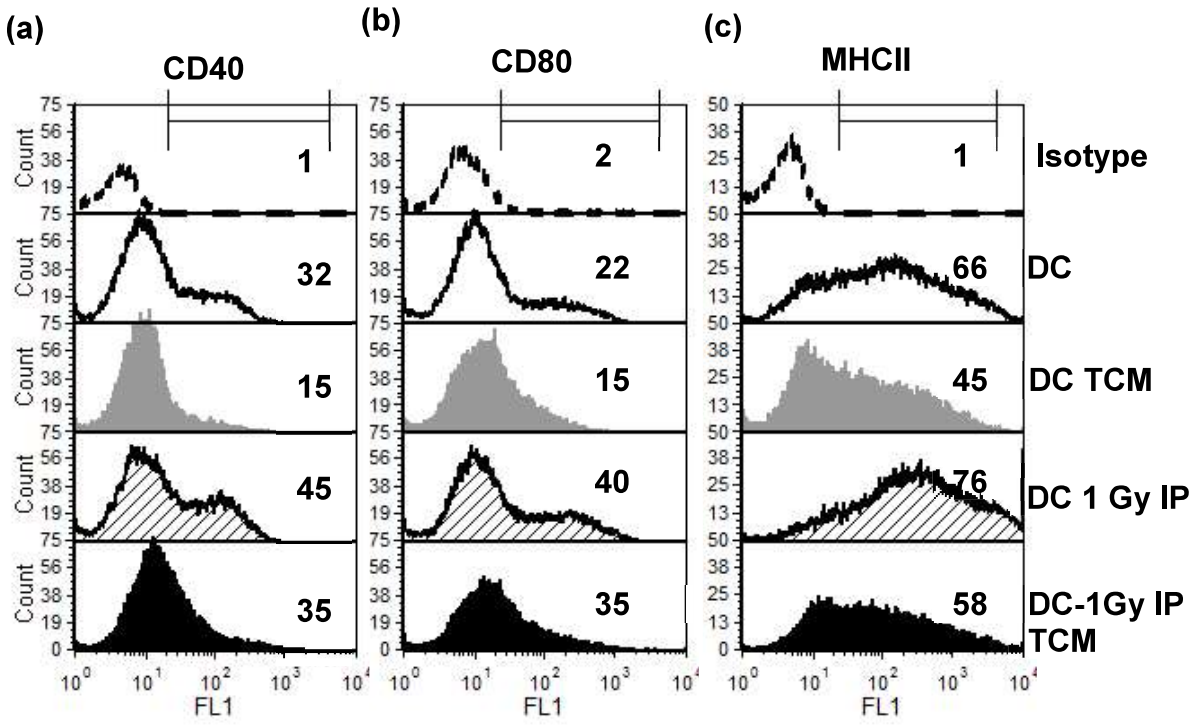


Fig 48: TCM induced suppression of DC maturation was not observed in DC generated from irradiated progenitors. BMC were isolated and exposed to 1 Gy irradiation. Cells were cultured for DC differentiation supplemented with TCM in 4 different sets: DC (control), DC (TCM), DC-1 Gy IP and DC-1 Gy IP TCM. Expression of (a) CD40 (b) CD80 (c) MHC II was analyzed using flow cytometry. Histograms presented are a representative figure from 2 independent experiments. Numbers in histogram represents percentage positive cells.

CHAPTER 4

DISCUSSION & CONCLUSIONS

4.1 Discussion:

Cancer is a group of disease which is characterized by uncontrollable growth and spread of abnormal cells. It is highly heterogeneous in nature, arises at different sites in the body and behaves differently depending upon the types of tissue, site of origin or type of mutation (*213, 214*). However this highly complex disease shares some fundamental aspects which are common to all types of cancer. Originally proposed by Hanahan and Weinberg, these common traits also known as “Hallmarks of cancer” includes sustained proliferative signaling, insensitivity to growth suppression, resisting apoptosis, sustained angiogenesis, replicative mortality and tissue invasion and metastasis. However, later two more hallmarks were added: reprogramming energy metabolism and evading immune response (*16*). Many recent studies exploring role of tumor microenvironment as well as immunodeficiency in tumorigenesis have corroborated the concept of cancer immune surveillance. Considerable evidence indicates that in order to progress, cancer has to breach the immune surveillance mechanism of body (*213, 215*). There are many different approaches through which cancer evade it, either directly by making immune system dysfunctional or indirectly by hiding itself from immune detection. In the context of cancer immune surveillance, role of dendritic cells become crucial to explore as they are the most important antigen presenting cells which has a unique capacity to take up exogenous tumor antigens and present it to cytotoxic T cell though ‘cross presentation’ for effective anti-cancer immune response (*65*). In order to avoid detection by dendritic cells, cancer cells exhibit different mechanisms including induction of apoptosis in DC, inhibiting DC maturation, manipulating DC development, generation of MDSC. However, it is not completely

understood how cancer cells affect the hematopoietic progenitor cells and its differentiation into different lineages. This becomes especially important since impaired progenitor cells means the differentiation to functional dendritic cells which will ultimately localize to the tumor will also be affected. Along with macrophages and erythrocytes, DC are differentiated from a common myeloid progenitor (CMP) (*206*). However, few DC subpopulations like $CD8^+$ $DEC205^+$ DC originate from lymphoid progenitors. Development of dendritic cells involves two distinct stages: Differentiation and maturation stage. Process of differentiation is a multi-step process and largely takes place in bone marrow. From CMP, a more restricted progenitor cell is differentiated inside bone marrow which is common precursor to monocytes, macrophages and classical DCs (Macrophage-DC progenitors, MDP). MDP is identified as $Lin^-CX3CR1^+CD11b^-CD115^+cKit^+CD135^+$. MDP further gives rise to DC specific common dendritic cell precursor (CDP) which is identified as $Lin^-CD115^+Flt3^+CD117^{Lo}$. CDP either gives rise to plasmacytoid DC (pDC) or classic DC (cDC) (*216*). These pDC and cDC further migrate to lymphoid or non-lymphoid organs through blood where the process of maturation takes place.

Like *in vivo* conditions, generation of BMDC under *in vitro* too involve differentiation and maturation stages. Results from our study indicate that tumor conditioned media or co-culture with tumor cells in trans well inserts has a very severe immunosupressive effect when it is present during differentiation process as compared to maturation process. This observation highlights the fact that differentiation is a multi-step process and different progenitors at successive differentiation processes show high plasticity, which also make them vulnerable to various immunosupressive factors present in tumor

microenvironment. As compared to differentiation, DC during maturation is terminally differentiated and less susceptible for external factors. Another distinct aspect of these two processes is that the changes occurring during differentiation are largely irreversible as compared to maturation process where it can be reversed. This is supported by the observation that when TCM induced dysfunctional immature DC were cultured in fresh media without TCM during maturation, there was no reversion of the phenotypic and functional dysfunction. This effect is in contrast with the effect of infectious agents like *M. tuberculosis* or HIV I on dendritic cells, where the changes are reverted once the pathogen load decrease (*217*). However similar differential effect of tumor microenvironment on either differentiation or maturation processes are not available in the literature. To the best of our knowledge, this is the first report of the differential effect of tumor microenvironment on differentiation and maturation processes of dendritic cell differentiation. On the other hand, it was demonstrated that downregulation of CD80/86 and MHC II by neural precursor cells (NPC) during differentiation could be reverted back during maturation upon removal of the NPC (*218*). These observations concur that changes that occur during late maturation phase are reversible in nature. However, changes that occur in differentiation phases are permanent and such functionality/dysfunctionality cannot be restored. This is supported by the observation that the once the macrophage dendritic cell progenitor (MDP) are differentiated into DC or macrophages based on selective cytokines (GM-CSF or M-CSF respectively) and they are committed into DC lineage after 2 days of culture with GM-CSF/IL-4, then they cannot be reversed even if these cytokines are removed. Generation of myeloid derived suppressor cells from early DC progenitor cells is another example which

highlights the restriction of plasticity in the differentiation process. Various cancer derived factors like GM-CSF, IL-10, TGFβ, VEGF generate immunosuppressive MDSC, which once generated does not revert to original progenitor phenotype even though immunosupressive tumor microenvironment is removed (*219, 220*). In addition, many secretory factors like IL-10, VEGF, TGFβ, nitric oxide and prostanoids, lactic acid, hyaluronan, reactive oxygen species have been identified in tumor microenvironment which negatively regulate DC function (*18, 155, 221-223*).

Many studies have explored the effect of tumor microenvironment on DC and identified various types of defects in DC functions. DC (TCM) generated in our study was both phenotypically and functionally defective. However, these two defects need not always be correlated with each other. Human DC isolated from head and neck cancer patients demonstrated defective antigen processing and presentation ability due to accumulation of lipids but did not show any downregulation of MHC II and other co-stimulatory receptors (*184*). Similarly, it was shown that tumor associated Treg cells affected the antigen processing and presentation ability of DC but did not affect the expression of CD40, CD86 and MHC II on DC (*224*). DC (TCM) also showed downregulation of allogenic T cells proliferation capacity which can be attributed to downregulation of MHCII expression.

In order to understand the active constituents present in TCM which was responsible for DC dysfunction, we evaluated the TCM for presence of immunosuppressive cytokines like IL-10 and TGFβ. but failed to detect their presence. Therefore, we estimated these cytokines in DC (TCM). IL-10 was detected in DC culture supernatant from day 6 onwards suggesting that TCM was inducing IL-10 secretion. IL-10 is a potent

immunosupressive cytokine and elevated levels of IL-10 has been found in many types of solid tumors and hematological malignancies (*225*). Serum IL-10 level has been widely explored as an independent prognostic factor in advanced solid tumors (*226*). Elevated levels of IL-10 could be a result of direct secretion from the cancer cells or by the immune cells (DC and Treg cells) due to the influence of cancer microenvironment. Irrespective of its origin, IL-10 negatively regulate DC function. DC that underwent maturation in presence of IL-10 switched the T_H1 type response into T_H2 *in vivo*, mainly due to downregulation of IL-12 (*210*). Tolerogenic DC that sustain expression of Foxp3 and TGFβ and promote Treg phenotype and functions are maintained in that state by autocrine activation of IL-10 receptor signaling (*227, 228*). These reports suggest that IL-10 affect both DC differentiation as well as maturation. In contrast, in our studies, when exogenous IL-10 was added on day 5 of DC culture, it failed to inhibit DC differentiation as observed in iDC on day 8. However addition of IL-10 during maturation phase did significantly affect the phenotypic maturation of DC. This could be due to the fact that addition of IL-10 was carried out in our study on day 5 of DC culture to mimic the effect of TCM, whereas in in those studies where IL-10 affected the DC differentiation it was present from day 0 (*229*). Induction of IL-10 on day 5 was accompanied with downregulation of IL-12 secretion by iDC. IL-10 and IL-12 are two characteristic cytokines associated with DC and their induction is mutually dependent on each other and the balance between IL-10 and IL-12 during their maturation process can influence DC to induce a T_H1 or T_H2 immune response (*210*). Autocrine IL-12 is necessary for IFNγ production by DCs that is centrally regulated by STAT4 (*230*). The IL-12-dependent STAT4 serine phosphorylation is mediated by stimulation of p38

mitogen-activated protein kinase (MAPK). How IL-10 induction inhibit IL-12 production is not conclusively known. In one study, it was shown that c-rel plays a central role in IL-10 induced inhibition of IL-12 secretion. IL-10 was shown to reduce the phosphorylation of IκBα, which ultimately resulted in poorer accumulation of c-rel in the nucleus as heterodimer of c-rel-NFkB complex (*231*). In another study, IL-10 was shown to induce ubiquitination and subsequent protein degradation of MyD88-dependent signaling molecules. Protein degradation by IL-10 was associated with decreased phosphorylation of p38, JNK, and IKK and ultimately resulted in decreased IL-12 production. Inhibition of protein ubiquitination /degradation was able to restore IL-12 production (*232*).

Our results demonstrate the involvement of ERK/CREB axis in TCM induced IL-10 induction by DC. CREB is a known regulator of IL-10, whose promoter element has a regulatory region where phosphorylated CREB binds and regulate a network of genes involved in metabolic processes (*208*). CREB could be activated upstream by either ERK1/2 or p38α MAPK pathways (*209, 233*). Inhibition of ERK1/2 phosphorylation inhibited CREB activation as well as intracellular IL-10 level confirming the TCM induced ERK/CREB axis of IL-10 induction. However inhibition of ERK did not restore TCM induced downregulation of phenotypic maturation markers. This observation suggested that IL-10 induction by TCM was not solely responsible for DC (TCM) dysfunction.

After ruling out the role of TCM induced DC secreted IL-10 in DC dysfunction, we further explored various other factors in TCM for their possible role in TCM induced DC immunosuppression. As we observed that IL-10 induction by TCM was guided by ERK-

CREB signaling axis, in this context various previous studies have suggested role of prostaglandins present in tumor microenvironment in induction of immunosuppressive IL-10. Prostaglandins have also been reported to directly affect DC differentiation (*234, 235*). When prostaglandin secretion was inhibited in cancer cells using specific COX-2 inhibitor NS-398 and tumor conditioned media derived from such cells were subsequently used during DC differentiation, we did not observe DC immunosuppression. This suggested possible role of prostanoids derived from cancer cells in DC dysfunction. Prostaglandins are known to regulate various metabolic as well as immune signaling pathways. PGE2 has been shown to be secreted by many types of cancer cells and actively participate in cancer progression (*236, 237*). Indirect role of prostaglandins in cancer progression comes from epidemiological cancer prevention studies which suggest that there may be a decrease in mortality from colorectal cancer in regular users of NSAIDs (*238*). Furthermore, another human study of familial adenomatous polyposis (FAP) revealed that administration of a specific COX-2 inhibitor (celecoxib) significantly reduced colorectal adenomas after 6 months (*239*). These epidemiological studies indirectly indicate the role of prostanoids in tumor promotion. A number of animal studies have also given direct evidence of PGE2 in tumorigenesis. It was shown that i.p. administration of PGE2 significantly increased the incidence and multiplicity of intestinal adenomas in F344 rats (*240*). Furthermore, *in vivo* studies have revealed that in familial adenomatous polyposis patients, the prevention of adenoma development is more effective when prostaglandin levels are reduced through NSAID treatment (*241*). However, the mechanism by which prostanoids help in tumor progression and particularly it's effect on the tumor microenvironment is not completely

understood. PGE2 increased epithelial cell proliferation mediated by the activation of the Ras-MAPK signaling cascade resulting in colon cancer in PGE2 treated mice (*242*). PGE2 pathway has been shown to influence the different hallmarks of cancer in conjugation with other pro-inflammatory factors. Evasion of apoptosis is one such hallmark of cancer which may be modified by COX-2. In one study it was shown that over expression of COX-2 led to elevated level of pro-survival protein B-cell lymphoma 2 (BCL-2) and it conferred increased resistance to butyrate-induced apoptosis in rat intestinal epithelial cells (*177, 243*). In other studies PGE2 has been shown to modulate various pro-survival pathways including the phosphoinositide 3-kinase/AKT (PI3K/AKT) pathway, ERK signaling, and epidermal growth factor receptor (EGFR) signaling (*244, 245*). PGE2 has also been suggested to have a role in hypoxia, where hypoxia inducible factor enhanced production of PGE2 which further help in survival of colorectal tumor cells (*246*). Overexpression of COX-2 has also been reported to induce expression of vascular endothelial growth factor (VEGF) and fibroblast growth factor (*247*). Together these factors are main components of angiogenesis, another hallmark of cancer which is reported to be regulated by PGE2 in some cancers.

Another hallmark of cancer is insensitivity to antigrowth signal such as transforming growth factor-beta (TGFβ), which blocks progression through the G1 phase of the cell cycle via the suppression of c-Myc and activation of cyclin-dependent kinase inhibitors (*248*). Cancer cells attain the insensitivity to TGFβ by different means and in one such mechanism, PGE2 has been shown to downregulate TGFβ receptor thus helping the cancer cells to maintain insensitivity to anti-growth signals (*243, 249*). Limitless replicative potential is another hallmark of cancer and it has been suggested that

colorectal cancer cells share the wingless-type MMTV (mouse mammary tumor virus) integration site (WNT) signaling pathway of progenitor cells in intestinal crypt. It was reported the connection between WNT signaling pathway and PGE2 signaling is mediated via β-catenin (*250*).

Metastasis is another hallmark of cancer which in some case is regulated by PGE2. It has been reported that inhibition of COX-2 *in vivo* can mitigate the metastatic potential of colorectal tumor in both humans and mice (*251, 252*). COX-2 was proposed as one of the four key signature genes that are involved in progression to metastasis (*187*). The report was based on the observation about essential role of PGE2 in metastasis of breast cancer cells to lung. Different mechanisms have been proposed to explain how PGE2 helps in metastasis and invasion. It was shown that through PI3K signaling, PGE 2 promotes cytoskeletal restructuring and thereby increases cancer cell migration and invasion (*244*). Furthermore, PGE2 has been shown to regulate transactivation of epidermal growth factor receptors through Src mediated signaling (*244, 253*). Through EGFR, PGE2 can also regulate hepatocyte growth factor/c-Met signaling which is associated with loss of cell to cell contact and metastasis (*254, 255*).

Though PGE2 has been reported to play an important role in regulating different hallmarks of cancer, it's effects in most of these cases is limited to maintenance of these hallmarks rather than initiation and establishment of these phenotypes. But in case of evasion of immune surveillance and anti-cancer immune response, PGE2 and other prostanoids play the central role. Antigen presenting cells especially dendritic cells are the most important mediator of immune surveillance as well as anti-tumor immune response. So the role of PGE2 in evasion of immune response is mainly

mediated through its effect on dendritic cells. PGE2 has both stimulatory and inhibitory effects on the DCs. It appears to have a stimulatory effect on DCs in peripheral tissues while in lymphoid organs where DC encounter antigens, PGE2 undertakes an inhibitory role, inhibiting the maturation of DCs and their ability to present antigen (*255, 256*). Many mechanisms have been postulated as to how PGE2 evades immune surveillance system through dendritic cells dysfunction. One of the first mechanisms of immunosurveillance system is the IFNγ production by dendritic cells at the site of inflammation. IFNγ serves as a chemoattractant to attract other antigen presenting cells and T cells which ultimately leads to control destruction of any mutated cells or to sublimate the inflammation (*257, 258*). It was shown that inhibition of IFNγ secretion by DC is modulated by PGE through inhibition of IL-12 secretion (*259*). IL-12 is the most critical factor for modulating the immune response towards a T_H1 type with a cytokine profile including IFNγ. In our study, we have reported decrease in level of IL-12 by DC (TCM) compare to DC control. This IL-12 downregulation could have been mediated through IL-10 upregulation or directly by PGE2 present in TCM or by both mechanisms. Many studies have provided evidence that PGE2 inhibit IL-12 production by dendritic cells. Incubation of DCs with PGE resulted in DC a phenotype that produced no IL-12 and high amounts of IL-10 (*259*). Such DC phenotypes promoted the development of T_H cells that produced high amounts of type 2 cytokine profiles especially IL-4 and IL-5. Prostaglandin E2 has also been reported as a selective inducer of interleukin-12 p40 (IL-12p40) production. Bioactive interleukin-12 p70 (IL-12p70) heterodimer is composed of two subunits of p35 and p40. In the absence of 12p35 subunit, 12p40 subunit form a homodimer. This homodimer along with free p40 monomer do not mediate IL-12 activity

instead act as IL-12 antagonists (*260*). This study suggested an additional level of the Th2-promoting activity of PGE2, via selective induction of IL-12p40 and suppression of bioactive IL-12p70.

In our study, exogenous addition of PGE2 during DC differentiation also resulted in immunosupressive phenotype of DC which confirmed the role of prostanoids/PGE2 in DC (TCM) dysfunction. Dendritic cells themselves secrete PGE2 and its effect has been shown to be crucial for DC maturation. This paradox of PGE2 effect on DC can be explained in terms of the timing, duration and amount of PGE2 secretion. PGE2 has a consistent inhibitory impact on early stages of DC development in contrast to its effect on the fully functional or immature DCs. In fact, PGE2 has been added along with the mixture of maturation cocktails of IL-1β and TNFα has been shown to accelerate DC maturation allowing their effectiveness at even 100-fold lower concentrations (*261*). In a similar experiment when PGE2 was used along with a defined cocktail of TNFα and IL-1β and IL-6, it enhanced the yield, maturation and immunostimulatory capacity of the DC generated compared to the cocktail without PGE2. In contrast to earlier report, where PGE2 has been shown to inhibit IFNγ secretion by DC, here it was reported that PGE2 treatment induced IFNγ secretion with no concomitant increase in IL-4 and IL-10 secretion (*262*). PGE2 treatment to DC has also been shown to increase the migratory potential of DC to lymph node. It was reported that in response to proinflammatory cytokine and CD40L, monocyte derived dendritic cells (MoDCs) acquired a proinflammatory cytokine secreting phenotype, which were non migratory. However in presence of PGE2 along with CD40L, MoDC attained a migratory phenotype with very less capacity to secrete cytokines (*263*). Both the migratory and non-migratory

proinflammatory DC expressed equivalent levels of chemokine receptors, which suggested the role of PGE2 in acquiring migratory phenotypes. However in contrast to this observation of no increase in CCR7 expression with PGE2, it has been shown that PGE2 addition directly upregulated CCR7 receptor on the cell surface of MoDCs (*264*). Most of these observations of PGE2 helping in DC maturation and migration was limited to the fact that PGE2 was added during maturation stages and not during differentiation. Also PGE2 was used in combination with other maturation agent and the effect was synergistic.

In our study, when bone marrow cells were isolated from tumor bearing mice and differentiated into dendritic cells under standard conditions *in vitro*, even such DC failed to differentiate into fully functional phenotype. This observation suggested that the effect of tumor microenvironment is not limited to DC or immune cells present in the microenvironment but that the progenitor cells which are distally located are also affected by the secretary mediators derived from the cancer cell. There are no reports in literature that explain the effect of cancer on bone marrow hematopoietic progenitor cells. The current understanding is that the tumor progression is not only guided by cancer cells and its interaction with other components of tumor microenvironment but through a complex systematic process also involving different cells/tissues present at a distant place (*265*). It was reported that $CD11b^{+}CD13^{+}$ myeloid cells constitute a population of bone marrow-derived cells that promote tumor progression and metastasis through their capacity to induce angiogenesis in solid tumor (*266*). These bone marrow cells express CD13, an aminopeptidase N which is a membrane-bound metalloprotease involved in pleiotropic functions including cell adhesion, proliferation. These cells also

secrete matrix metalloproteinase (MMP)-9 and osteopontin (OP) which are known to play role in angiogenesis (*267*). In a recent study, it was shown that insulin-like growth factor 2 secreted by inhibitor of differentiation (Id1)-overexpressing esophageal cancer cells induce VEGFR1-positive bone marrow cells which after induction form pre-metastatic niches at distant sites by increasing VEGF secretion (*233*). These metastatic niches are then used as dock to attract cancer cells via CXCL5/CXCR2 axis. CXCL16 expressed by prostate cancer have also shown to attract mesenchymal stem cells expressing CXCR6. These stem cells further through CXCR6 signaling converted into cancer associated fibroblast and help in progression of cancer through various mechanism (*268*).

Bone marrow consists of different lymphoid and myeloid progenitor cells along with erythrocytes and other stromal cells. The differentiation of these progenitor cells is also regulated by a complex network of transcriptional factors. Very recently, transcriptional network which regulate the dendritic cells lineage and differentiation has been deciphered under the aegis of the Immunological genome project (*269*) The study identified various signature transcription factors for different specific lineages of DC. Our study for the first time reveals that bone marrow cells isolated from tumor bearing mice showed downregulation of Zbtb46 and Bcl6 transcription factor. Zbtb46 is a signature transcription factor for classic DC lineage which is not expressed by plasmacytoid DC. Satpathy et al 2012 first reported that Zbtb46 is exclusive to cDC lineage (*263*). They replaced the first coding exon of Zbtb46 with GFP using homologous recombination and showed with GFP expression that Zbtb46 expression was restricted to either pre-cDCs, lymphoid organ- and tissue-resident cDCs. Although Zbtb46 deficient mice showed

development of cDC, such cells were not functional and showed expression of leukemia inhibitory factor receptors, normally down-regulated in cDCs. In a similar experiment, diphtheria toxin (DT) receptor (DTR) cDNA was introduced into the 3′ UTR of the Zbtb46 locus and introduction of DT injection into zDC-DTR bone marrow resulted in cDC depletion (*121*).

The role of Zbtb46 in tumor induced DC dysfunction has not yet been identified. In our study, we observed that Zbtb46 knockdown in bone marrow progenitor cells resulted into immunosupressive phenotype of BMDC like DC (TCM). This observation confirmed our hypothesis that TCM induced DC dysfunction was mediated through downregulation of Zbtb46. Exogenous addition of PGE2 in BMC culture also reduced Zbtb46 expression in BMC similar to TCM and the downregulation was mitigated with blocking of EP2 receptor. TCM induced secretion of IL-10 as well as downregulation of Zbtb46 are involved in DC dysfunction. However blocking of ERK signaling in DC (TCM) could mitigate only the upregulation of IL-10 but not the downregulation of Zbtb46 which suggested that these two events may be triggered by prostanoids in tumor microenvironment but their downstream signaling could be different.

In our studies, use of NS-398 not only rescued the DC (TCM) dysfunction under *in vitro* condition but also in splenic DC of TBM. NS-398 was first reported by N.Futak et al in 1994 as a selective inhibitor of COX-2, the inducible form of cyclooxygenase (*270*). IC50 of NS-398 was found to be 3.8 μM while COX-1 activity was completely unaffected by NS-398 even at 100 times more concentration. Through COX-2 inhibition, NS-398 has been shown to be useful in various pathophysiological conditions including cancer. It was shown that choroidal neovascularization (CNV) lesion, an important pathological

component in autoimmune muscular degeneration (AMD) was attenuated by the administration of NS-398 (*271*). Similarly intrathecal administration of NS-398 in rat modulated the flinching behavior in a dose-dependent manner in spinal nociceptive transmission (*272*). In cerebral ischemia, COX-2 play a role in post-ischemic inflammation and mediates the ischemic brain injury. It was shown that NS-398 inhibited the inflammation and associated neurotoxicity associated with it (*273*). In contrast to the pro-inflammatory role of COX-2 in cancer, many studies have reported anti-tumor properties of NS-398. NS-398 induced apoptosis in different hepatic cell lines (*274*). Similarly COX-2 expression was also correlated with high grade gliomas and NS-398 inhibited the proliferation, spheroid formation and migration in human glioblastoma cell lines (*275*).

In our study we have shown that NS-398 treatment significantly lowered PGE2 levels in tumor bearing mice and there was a concomitant upregulation in Zbtb46 in bone marrow progenitor cells, which further translated into improved phenotypic maturation of splenic DC. Earlier studies have associated the serum PGE2 level with dendritic cell dysfunction. We also observed that NS-398 treatment did not have any effect on tumor volume in tumor bearing immunocompromised SCID mice. Although there were improvement in phenotypic maturation of splenic DC, it could not be translated into anti-tumor immunity. SCID mice lack both the T and B lymphocytes and in the absence of effector cells, any improvement in dysfunctional DC has no meaning. Based on anti-tumor effect of NS-398, we have also used a combinatorial approach in which camptothecin, a known cytotoxic drug has been administered along with NS-398. Camptothecin is an effective anti-tumor drug. Camptothecin along with its analog such

as topotecan, are used in the therapy of ovarian, cervical, colorectal and small cell lung cancers (*276*). It has increased survival time in mice bearing different leukemia (*277*). However one of the main issue with camptothecin and its analogue is its cytotoxicity to normal cells at doses witch are essential for effective anti-tumor activity. Our objective was to find out if there is any synergistic effect of NS-398 along with camptothecin which can lead to a probable dose reduction. In our study, we observed that reduction in tumor volume in TBM (NS-398+CPT) group was much more significant than any of them alone. It has been reported that in case of mesothelioma cancer, addition of NS-398 or Dip-697 (COX-2 inhibitor) increased the cytotoxic effect of premetrexed, a known FDA approved chemotherapy drug. In this study three different mesothelioma cell lines MSTO-211H, NCI-H2052, NCI-H2452 were treated with premetrexed along with NS-398 and it was observed that there was 30 to 40 fold decrease in IC_{50} value of premetrexed in combination with NS-398 (*278*). In similar study, celecoxib, and indomethacin had increased the apoptotic effect of docetaxel and cisplatin in A549 cells (*279*).

Inhibition of cyclooxygenase and cardiovascular risk has long been debated. Studies associated the use of celecoxib in a clinical trial with 2035 patients for colorectal adenoma prevention with increase in risk of serious cardiovascular events in a dose dependent manner (*280*). However, conclusion from these studies is debatable and it is not known that cardiovascular risk of COX inhibitor is drug specific or by virtue of being COX inhibitor. A long-term arthritis safety study (CLASS) with celecoxib (400 mg twice daily), did not show any increased rate of cardiovascular events compared to two nonselective nonsteroidal anti-inflammatory drugs (NSAIDs) (*281*). The studies exploring the cardiovascular effects of COX inhibitors are not conclusive and many

other factors like geographical differences, genetic predisposition, and concomitant medications may be playing role thus resulting in contrasting reports.

Our observation about role of Zbtb46 transcription factors in tumor induced DC dysfunction open up a new question about how Zbtb46 is regulated in DC. Not much is known about regulation of lineage specific transcription factors in immune cells. Various studies have shown the importance of miRNA in regulating the transcriptional network of an immune cell especially under stress conditions including inflammation and cancer (*282, 283*).

In this study, we compared the differential global expression of miRNAs in dendritic cells with and without tumor microenvironment through RNA sequencing. Based on the RNA sequencing data and the literature available we confirmed that miR-155p, miR-155-3p and miR146a-5p were found to be upregulated in DC (TCM) as compared to DC. miR-155 family is crucial regulators of innate immunity and inflammatory responses and play a very significant role in immune regulation. It has been shown that miR-155 repressed SOCS1 critical for LPS-driven TLR signaling, which regulate endotoxin sensitivity and tolerance (*284*). It was further shown in macrophages that miR-155 directly targets transcripts which code for proteins involved in LPS signaling such as the Fas-associated death domain protein (FADD), IkB kinase (IKK-ε), while enhancing TNFα translation (*285*). Analysis of miR155-deficient dendritic cells demonstrated that miR-155 is required for DC maturation and for the ability of DCs to promote antigen-specific T-cell activation through silencing of transcription factor Fos (*137, 285*). Increased miR-155 expression induced apoptosis in dendritic cells mediated by accumulation of p27kip1, a cyclin-dependent kinase (*286*). Also the mDCs from miR-

$155^{-/-}$ mice underwent less apoptosis as compared to those from wild-type mice. Although there are reports which also suggested that miR-155 may display anti-oncogenic or pro-immunological role (*163*), in general miR-155 has been implicated in promoting many types of cancers including breast, lung, liver, and lymphatic system(*287*). Various mechanism has been proposed how miR-155 help in promotion of cancer. It was shown that miR-155 was essential for the accumulation and function of myeloid-derived suppressive cells (MDSC) in the tumor microenvironment. miR-155 not only helped in induction of MDSC but also it was also essential for the MDSC-mediated $CD4^{+}Foxp3^{+}$ regulatory T cells (Treg) induction (*288*). How TCM induced upregulation of miR-155 in DC helps in tumor promotion is not clear. In our study we have observed upregulation of miR-155 not only in iDC (TCM) compared to iDC but also in mDC compared to iDC. Earlier reports have also suggested the role of miR-155 in induction of maturation in DC (*137*). Together these studies suggest a hypothesis that tumor microenvironment may induce early maturation in DC and thereby make DC either tolerogenic or dysfunctional. These results are therefore in agreement with reports which have suggested that tumor microenvironment induce early maturation of dendritic cells which further leads to apoptosis (*162*).

In this study we have also observed upregulation of miR-146a-5p in iDC TCM as compared to iDC. miR-146a regulate the functional heterogeneity of monocytes by directly targeting Relb, a member of the noncanonical NF-κB/Rel family (*289*). Decreased miR-146a levels also been shown to be correlated with increased gene expression of proinflammatory target genes (*290*), suggesting an anti-inflammatory role of miR-146a. One of the most critical role of miR146a is regulation of suppressive

function of Treg cells. It was reported that the deficiency of miR-146a in Treg cells resulted in a breakdown of immunological tolerance (*291*). It was shown that miR-146a directly targets the signal transducer and activator transcription 1 (Stat1). STAT1 also play critical role in DC activation and maturation by IL-4 and GM-CSF, known to induce DC differentiation. Role of miR-146a has already been reported in dendritic cells. It was demonstrated that the expressions of myelogenic miRNAs such as miR-155, miR-146a, miR-146b, miR-142-5p were increased in aged BMDC and there was concomitant decrease in production of pro-inflammatory cytokines in aged BMDC (*139*). Increased expression of miR-146a has also been shown to increase apoptosis in dendritic cells. When miR-146a was overexpressed in DC, expression of TRAF6/IRAK1 was reduced which further upregulated NFkB inhibitor and decreased Bcl-2 expression, a known suppressor of apoptosis (*139*). Our study has also found downregulation of miR-187-3p and miR-365-2-5p. No conclusive role of these miRNA has so far been reported in dendritic cells. However miR-187 has been shown as prognostic factor in breast cancer. In silico coinertia analysis was used to show that miR-187 expression was associated with reduced breast cancer specific survival (*292*). Anti-tumor role of miR-187 has also been studied. In hepatocellular carcinoma, miR‑187 acted as a tumor suppressor by direct targeting and downregulating IGF‑1R expression. Restoration of miR‑187 expression inhibited cell proliferation, migration and invasion in HCC (*293*). Similarly expression of miR-187 was downregulated with a consequent negative regulation of TGFβ signaling in colorectal cancer (*294*). Furthermore, it was observed that miR-187 directly target the expression of SRY-related HMG-box4 (SOX4), 5'-nucleotidase (NT5E) and Protein-tyrosine kinase 6 (PTK6), which were essential upstream effectors

of Smad pathway. miR-187 also suppressed cancer cell progression in non-small cell lung cancer (NSCLC) through down-regulation of Cytochrome P450 1B1 (CYP1B1) (*295*). miR-187 has been shown to be induced by IL-10 where it negatively regulates TNFα, IL-6, and IL-12p40 production in TLR4-stimulated monocytes (*296*). In our study we have observed significant downregulation of miR-365-2-5p. In cutaneous squamous cell carcinomas, expression of the miR-193b/365a cluster was found to be downregulated during tumor progression suggesting a possible tumor suppressor role . Ectopic expression of miR-365a in tumor cells inhibited their proliferation, clonogenic potential and migration ability (*297*). It has also been found to negatively regulate IL-6 gene expression (*298*). When an in silico analysis was performed using miRTargetLink Human (https://ccb-web.cs.uni-saarland.de/mirtargetlink/) for prediction of possible interaction between miRNAs of interest identified in our studies, it was found that miR-155 and miR-146 shared 6 common targets in human transcriptome. These include SMAD2, SMAD4, ICAM1, RAC1, IL-8 and FADD.

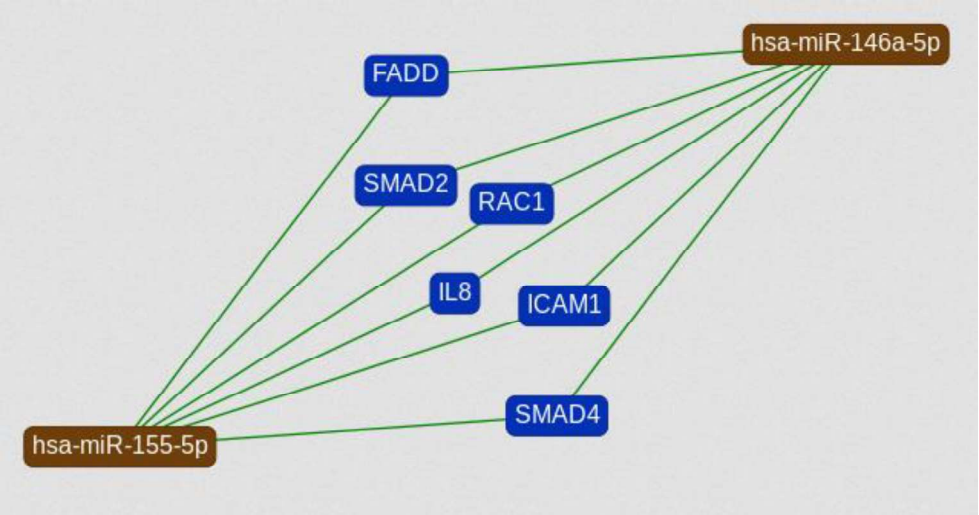


Fig 49: **Schematic representation of target genes overlaps of hsa-miR-155 and hsa-miR-146a (miRTargetLink Human analysis).**

FADD is fas-associated protein with death domain (FADD), is an adaptor protein that bridges Fas-receptor, to procaspases 8 and 10 to form the death-inducing signaling complex (DISC) during apoptosis. Both miR-155 and miR-146a has been independently shown to modulate activation-induced cell death, acting as an antiapoptotic factor, by directly targeting Fas-associated death domain (FADD) (*299, 300*). Upregulation of miR-146a and miR-155 in dendritic cells under tumor microenvironment may increase the life span of dendritic cells and which in turn can induce a tolerogenic phenotype of dendritic cells (*301*). SMAD2 and SMAD4 are also potential targets of both miR146a and miR155. SMAD2 and SMAD4 both form heterodimer. This complex is known to regulate many transcription factors involved in TGFβ signaling. They are considered to be tumor suppressive in nature (*302*). Bioinformatic analysis also reveals ICAM1 as potential target of miR-146a and miR-155. ICAM is a glycoprotein important for cellular adhesion. It is expressed on monocytes and follicular DC in response to many inflammatory

mediators, such as IFNγ and TNFα (*303*). How targeting of ICAM in DC would play out is inconclusive and needs to be explored.

Our observation that tumor microenvironment affects the progenitors of dendritic cells could probably explain why DC based immunotherapy has not been so successful. In most of DC immunotherapy protocol, CD14 monocytes are isolated from blood of the patient and *ex vivo* differentiated into DC for further processing. However if the tumor microenvironment affect the early differentiation from progenitor to monocyte, then these cells would be inherently incapable to incite an effective anti tumor immune response. Treatment with COX-2 inhibitor is one such strategy to mitigate the DC dysfunction. There are reports which suggest that radiotherapy could also induce dendritic cells for better processing and presentation.

Radiotherapy is one of the most important treatment modality for cancer. It induces either apoptotic or necrotic cell death in cancer cells as well as tumor infiltrating immune cells and surrounding cells (*304*). Owing to hyper sensitivity of lymphocytes like T cell and B cells, radiotherapy is also considered as immunosuppressive or tolerogenic in nature (*305*). However recent studies about the effect of radiation on immune system has suggested that radiation is more of an immunomodulator than an immunosuppresor. These immunomodulation activities of radiation include downregulation of regulatory T cell activity, upregulation of tumor-associated antigens, MHCI molecules, increasing cancer cell sensitivity against CTL and activate antigen presenting cells such as dendritic cells through TLR dependent mechanisms (*306-308*). Post irradiation analysis also revealed increased expression of proinflammatory cytokines, including TNFα and IL-1β (*309, 310*). Basically the immunomodulatory role of

radiation is due to irradiation induced ‘danger signals’ from necrotic tumor cells which further incite a potent antitumor immune response and revert the immunosuppressive tumor microenvironment. Dendritic cells are considered as highly radio-resistant compared to other immune cells as they are terminally differentiated in nature. However, irradiation of dendritic cells have shown to downregulate antigen processing ability (*186*), induce migration of dermal and epidermal dendritic cells (*311*), downregulate T cell proliferation capacity of dendritic cells (*312*), and also downregulate expression of co-stimulatory molecules like CD80/86 on dendritic cells (*313*). Both the immunomodulatory effect of radiation and function of dendritic cells converge through danger signal hypothesis. According to this hypothesis, dying cancer cells released many molecules which are identified by antigen presenting cells as danger signals through innate immune receptors like toll like receptors. This further induces an immune response against cancer cells. It was reported that after radiotherapy, dying tumor cells released high mobility group box 1 protein (HMGB1). HMGB1 interacted with TLR4 on DCs, which were involved in the cross-priming of anti-tumor T lymphocytes *in vivo* (*190*). Similarly calreticulin was identified as another mediator where translocation of calreticulin to the surface of dying cancer cells facilitated their uptake by dendritic cells (*211*). Another study demonstrated Hsp70 mediated dendritic cell maturation and anti-tumor immune activation when colorectal tumor cells were treated with X-rays and hypothermia (*314*). In our study bone marrow progenitor cells were irradiated and BMDC generated from irradiated precursor cells showed increase in phenotypic and functional properties. When apoptotic cells were removed, 24 hr after irradiation increase was abrogated. This observation suggest possible role of apoptotic cells in this

phenomenon. We did not observe any upregulation in HMGB1 and calreticulin in irradiated bone marrow cells as compared to control. However we observed decrease in Hsp70 in irradiated cells, probably due to release of Hsp70 outside the cells. This result, however, need further exploration to draw any conclusion. Bone marrow cells from whole body irradiated mice also showed increase in apoptosis and subsequent increase in mature phenotype of dendritic cells. It has been suggested that gamma irradiation regulates the level of cytokine-mediators through transcriptional modulation, including signal transducer and activator of transcription (STAT) phosphorylation. Different members of STAT signaling is involved in DC differentiation and maturation (*315*). STAT5 is known to regulate classic DC lineage and STAT3 is considered as regulator of plasmacytoid DC through lineage specific transcription factors Id2 and E2-2 respectively (*212*). We have observed upregulation of STAT5 phosphorylation and downregulation of STAT3 phosphorylation in DC from irradiated precursor cells. We have also observed upregulation of Zbtb46 transcription factor as well as Id2 transcription factor. Both these transcription factors regulate classic $CD103^{+}$ DC differentiation. Both these observations suggest that irradiation of bone marrow progenitors could possibly direct the DC differentiation more towards classic DC. We have also reported that irradiated progenitor cells when cultured with tumor conditioned media from EL4 lymphoma, did not show suppression to the same extent as DC (TCM) differentiated from control progenitor cells.

Although the mechanism of this phenomenon is not known, it suggests possible convergence of TCM induced DC dysfunction as well as irradiation induced increase in DC immunogenicity. These results therefore suggest possible activation of pathways

that may be antagonistic to PGE2 signaling. The clearance of apoptotic cells by tissue macrophages, dendritic cells and nonprofessional phagocytes is an essential process in tissue homeostasis, immunity, and resolution of inflammation. Though no literature reports are available on the effect of apoptotic cells on bone marrow cells undergoing differentiation process, it was reported that macrophages following exposure to apoptotic cells upregulate COX-2/PGE2 and hepatocyte growth factor (HGF) expression via a positive feedback loop (*316*). Though there is no direct evidence, we can speculate similar activation of some growth factor signalling pathway by a feedback loop which has made these cells refractive to further action of PGE2 (*317*). Another possibility is that radiation induced expression of EP2 receptor. Prosurvival and antiapoptotic effects of PGE2 in radiation injury are mediated by EP2 receptor in intestine which is known to antagonize PGE2 effects (*318*).

The findings from this dissertation thus highlight the vulnerability or plasticity of the progenitor cells that decides the outcome of its differentiation into dendritic cells. On one hand, the soluble factors, prostanoids secreted by tumor cells could irreversibly alter the differentiation potential of progenitors. On the other, the apoptotic cells in the microenvironment could increase the potential of the progenitors to differentiate into dendritic cells. Not only that, these DCs generated from irradiated progenitors were refractory to the effects of tumor induced suppression or in other words had activated pathways that were antagonistic to prostanoid signalling. These studies thus reiterate that the soluble mediators present in the tumor microenvironment not only have the capacity to alter the fate of cells in the milieu but also the distal cells and that too irreversibly. These studies reveal that these changes in the progenitor cannot be altered

and can only be averted by neutralising the original source or the soluble mediator itself that is responsible for the effects. The results reported in this thesis have also given a glimpse regarding the effect of how routinely used therapeutic modality like radiation also can affect the differentiation status of DC. The identification that inhibition of prostanoid synthesis can be effective along with cytotoxic drugs has tremendous potential in cancer treatment to reduce the cytotoxic drug dosage by inclusion of a COX-2 inhibitor in the therapeutic regimen. Since specific COX-2 inhibitors like celecoxib have been shown to reduce adenomas in patients (223), it would be interesting to study if there was an involvement of dendritic cells. It would be also pertinent to study if Zbtb46 or the miRNAs identified in this study can be used as a reliable biomarker to detect the functional capacity of DCs in cancer patients.

4.2 Summary:

Cancer cells evolve various mechanisms to grow and evade immune surveillance system of body and cancer microenvironment plays an important role in this. Being the only antigen presenting cells that have ability to cross present tumor antigens to cytotoxic T cells, dendritic cells are one of the prime target of cancer microenvironment. The most important finding our study is that the tumor microenvironment makes DC dysfunctional by affecting the early differentiation process of DC from progenitor cells and these changes are irreversible. Tumor conditioned media inhibited the phenotypic and functional maturation of DC both *in vitro* and *in vivo*. TCM, when present during DC differentiation, induced secretion of IL-10 cytokine through ERK-CREB signaling axis. However inhibition of IL-10 by inhibiting ERK phosphorylation was not able to mitigate the TCM induced downregulation of DC function, suggesting that IL-10 was not the prime reason for DC immunosuppression.

Analysis of lineage specific transcription factors of DC in cancer microenvironment was also carried out to evaluate their role in DC dysfunction. We observed that myeloid lineage specific transcription factor Zbtb46 was consistently decreased in DC (TCM) and DC (TBM) as compared to respective control. To ascertain whether Zbtb46 downregulation is the reason of DC dysfunction, Zbtb46 was knocked down in DC during early differentiation as well as maturation. Knock down of Zbtb46 during maturation did not affect the DC phenotype significantly however early knock down of Zbt46 significantly affected the DC phenotypic maturation similar to TCM.

Prostanoids were identified as the main constituents of tumor microenvironment causing DC dysfunction, as TCM derived from EL4 cells cultured with NS-398 (COX-2 inhibitor) did not have similar effects as TCM. Further when exogenous PGE2 was added into DC culture during differentiation, it resulted in similar DC phenotype as DC (TCM). Also exogenous PGE2 downregulated Zbtb46 T.F. in DC as well as DC progenitors.

In order to study the *in vivo* implication of our finding, tumor bearing mice model were generated and treated with NS-398. We observed that NS-398 treatment was able to downregulate PGE2 level in mice serum. Zbtb46 expression and splenic DC maturation was restored and subsequently tumor burden in TBM (NS-398) was reduced by 54% when compared to TBM (control) ($p=0.008$) and 52% when compared to TBM (vehicle) ($p=0.01$). Similar treatment of NS-398 in lymphoma model generated in immuno-compromised SCID mice did not result in reduction in tumor volume, although DC maturation was relatively improved. In order to evaluate the anti-tumor effect of a combinatorial therapeutic modality, we used camptothecin (cytotoxic drug) along with NS-398. We did observe significantly improved reduction in tumor volume with CPT-NS-398 combination as compared to CPT alone ($p=0.011$) or NS-398 alone ($p=0.004$).

Role of miRNAs was evaluated in DC in context of cancer microenvironment. Gobal miRNA sequencing analysis was carried out in different treatment groups. iDC (TCM) showed significant differential regulation of 9 miRNAs as compared to iDC ($p<0.05$). Comparison between iDC and mDC showed significant differential regulation of total 29 miRNAs including 3 novel miRNAs which have not been annotated yet. From the list of iDC vs iDC TCM comparison, we chose 6 miRNA (miR-155-5p, miR-155-3p, miR-146a-3p, miR-365-2-5p, miR-187-3p, miR-142a-3p) and validated their expression profile

through RT PCR analysis. miR-155-5p, miR-155-3p and miR-146a-3p showed upregulation of expression in DC (TCM) as compared to iDC both in RNA-seq data as well as RT PCR validation. miR-365-2-5p and miR-187-3p also followed the similar pattern of downregulation in RT PCR validation as in RNA seq analysis. miR-142a-3p expression showed a different pattern.

Recent studies have indicated the immunomodulatory effect of chemo and radiotherapies. In this context, we studied the effect of radiation on DC differentiation and maturation. We observed that irradiation of bone marrow cells and subsequent differentiation of BMDC from irradiated progenitor resulted in DC with improved phenotypic maturation and functional properties like cross presentation ability. There was no change in phagocytic ability of DC. Also DC from irradiated precursor cells secreted more IL-12 and TNFα as compared to DC-CP. The effect of irradiation was not dose dependent. We observed that removal of apoptotic cells 24 h after irradiation through density gradient centrifugation abrogated the irradiation induced increase in immunogenicity, suggesting role of apoptotic cells. However contrary to previous studies we did not observe change in the level of HMGB1, calreticulin, although Hsp70 was found to be decreased after irradiation. BMDC generated from BMC isolated from WBI mice also showed upregulation of phenotypic maturation.

4.3 Conclusion:

Conclusions from this Ph.D. thesis are as follows:

- Cancer microenvironment induced dysfunction in dendritic cells in *in vitro* and *in vivo* condition both at phenotypic level and functional level.
- TCM induced secretion of immunosuppressive IL-10 by BMDC through ERK-CREB signalling pathway.
- Inhibition of ERK activation inhibited CREB phosphorylation and decreased IL-10 production, however it did not mitigate DC dysfunction.
- rIL-10 when added during DC differentiation did not significantly affect DC immunogenicity like TCM. Inhibitory effect was evident when rIL-10 was present during maturation.
- BMDC generated from tumor bearing mice showed phenotypic and functional dysfunction.
- Expression profile of lineage specific transcription factors showed downregulation of Zbtb46 and Bcl6 in DC due to cancer microenvironment both *in vitro* and *in vivo*.
- TCM (NS-398) did not show similar effect on DC as TCM. Addition of PGE2 to DC culture downregulated maturation markers, Zbtb46.
- NS-398 treated TBM showed restoration of DC function and reduction in tumor burden while NS-398 treatment in tumor bearing SCID mice did not show tumor reduction.

- NS-398 along with CPT showed better anti tumor response than NS-398 or CPT alone.
- DC and BMC showed differential expression of various miRNAs in cancer microenvironment.
- Both iDC (TCM) and mDC showed upregulation of miR-155 and down regulation of miR-365-2 as compared to iDC, suggesting the possibility that TCM induced early maturation in DC.
- BMDC generated from irradiated precursor showed increase in phenotypic and functional maturation.
- Increase was abrogated when apoptotic cells were removed from culture.
- BMC from WBI mice showed dose dependent increase in apoptosis with concomitant increase in maturation of DC.
- Irradiation of progenitor cells significantly mitigated the TCM induced DC suppression.

4.4 Future prospects:

- Study the upstream regulation as well as the downstream targets of Zbtb46 transcription factor in dendritic cells.
- To further explore the role of lineage specific transcription factors of DC in cancer microenvironment.
- *In vivo* standardisation of dose, duration and pharmacokinetics and pharmacodynamics study of cytotoxic drugs along with NS-398.
- Structural modification of NS-398 to increase its solubility and bioavailability *in vivo*.
- Screening and identification of more COX-2 inhibitors with better efficiency with less side effects than NS-398.
- To explore the role and consequences of differentially expressed miRNA in DC in cancer microenvironment identified through RNA seq using silencing and overexpression studies.
- Validation and annotation of novel miRNAs identified as differentially regulated in DC (TCM) and TBM BMC.
- To explore the possible connection between miRNA identified in our studies and Zbtb46 regulation.
- Conclusive identification of active component of apoptotic progenitor cells responsible for increase in DC immunogenicity.
- To explore the clinical implication of radiation and its impact on DC differentiation especially in cancer microenvironment.

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ANNEXURE I:

Materials:

Chemical name	**Source**	**Catalog no.**
2-Mercaptoethanol	Sigma (St. Louis, MO, USA)	M6250
Acrylamide		A3553
Ammonium Sulphate		A4418
Bovine Serum Albumin		A2153
Bradford reagent		B6916
Dimethylsulfoxide (DMSO)		D2650
Ethylenediaminetetraacetic acid(EDTA)		E6758
Glycine		G8898
Gycerol		G5516
HRP chemiluminescent substrate	Millipore (Billerica, MA, USA)	WBKLS0500
Igepal (NP-40)	Sigma (St. Louis, MO, USA)	I7771
Non-fat milk	HiMedia (Mumbai, India)	M530
PD 98059	Sigma (St. Louis, MO, USA)	P215
Phosphatase inhibitor cocktail tablets	Roche Applied Science (Germany)	4906 837 001 262

PI	Sigma (St. Louis, MO, USA)	P4170
Ponceau S solution		P7170
Pre-stained Molecular weight marker	Bio-Rad (Hercules, CA, USA)	161-0325
Protease inhibitor cocktail tablets	Roche Applied Science (Germany)	11 873 580 001
PVDF membranes	Millipore (Billerica, MA, USA)	1PVH00010
SDS	Sigma (St. Louis, MO, USA)	L3771
Sodium azide		S2002
Sodium bicarbonate		S5761
SYBR green	Roche Applied Science (Germany)	S9430
TEMED	Sigma (St. Louis, MO, USA)	T9281
Tris base		T1503

TrisHCl		T3253
Triton X 100		X100
Tween 20		P2287

Annexure II:

Antibody and assay kits:

Name of Antibody	**Source**	**Catalog no.**
Anti mouse CD40 (clone 3/23)	BD Biosciences ((Franklin Lakes, NJ, USA)	553788
Anti mouse CD80 (clone 1G10),	BD Biosciences ((Franklin Lakes, NJ, USA)	553368
Anti mouse CD86 (clone PO3)	BD Biosciences ((Franklin Lakes, NJ, USA)	553689
Anti mouse IA/IE (clone 2G9)	BD Biosciences ((Franklin Lakes, NJ, USA)	553621
Anti mouse CD3 (clone Clone 17A2)	BD Biosciences ((Franklin Lakes, NJ, USA)	555273
Anti mouse CD4 (clone RM4-5)	BD Biosciences ((Franklin Lakes, NJ, USA)	550280
Anti mouse CD8a (clone	BD Biosciences ((Franklin	550281

53-6.7)	Lakes, NJ, USA)	
Anti mouse CD11c (clone HL3)	BD Biosciences ((Franklin Lakes, NJ, USA)	550283
Anti mouse CD19 (clone 1D3)	BD Biosciences ((Franklin Lakes, NJ, USA)	550284
Anti rat IgG Alexa fluor 488	BD Biosciences ((Franklin Lakes, NJ, USA)	
PE-Anti mouse IL-2 (clone JES6-5H4)	BD Biosciences ((Franklin Lakes, NJ, USA)	554428
FITC Anti mouse IL-10 (clone JES5-16E3)	BD Biosciences ((Franklin Lakes, NJ, USA)	554467
PE-D1.16	eBiosciences (SanDiego, CA, USA).	12-5743-82
Anti mouseZbtb46/BTBD4 (clone G-15)	Santa Cruz Biotechnology (Santa Cruz, CA)	SC-85312
Anti-mouse EP-2 (H-75),	Santa Cruz Biotechnology (Santa Cruz, CA)	SC-20675
Anti-mouse EP-4 (C-4)	Santa Cruz Biotechnology (Santa Cruz, CA)	SC-55596
pERK1/2 (Thr 202/Tyr 204)	Santa Cruz Biotechnology (Santa Cruz, CA)	SC-16982
Anti mouse SNFT/Bcl6 (clone M-13)	Santa Cruz Biotechnology (Santa Cruz, CA)	SC-162246

p-CREB-1 (Ser 133)	Santa Cruz Biotechnology (Santa Cruz, CA)	SC-7978
Mouse IL-12	BD Biosciences (Franklin Lakes, NJ, USA)	555256
Mousse IL-10		555252
Mouse TNF		555268
Prostaglandin E2 ELISA- Monoclonal	Cayman chemical, Ellsworth Rd · Ann Arbor, MI · USA	514010
Mouse rGMCSF	Miltenyi Biotech, Bergisch Gladbach, Germany	130-095-739
Mouse rIL-4		130-097-757
CD11c microbeds		130-108-338
CD4 (L3T4) microbeads		
rIL-10	Sigma (St. Louis, MO, USA)	I3019
PGE2	Sigma (St. Louis, MO, USA)	P5640
NS-398	Sigma (St. Louis, MO, USA)	N194
siRNA pool (Zbtb46)	Dharmacon GE lifesciences (Lafayette, CO, USA)	
siRNA pool (Bcl6)	Dharmacon GE lifesciences (Lafayette,	

	CO, USA)	
LightCycler 480 SYBR Green master mix	Roche (Penzberg, Upper Bavaria, Germany)	04887352001
X-tremeGENE siRNA Transfection	Roche (Penzberg, Upper Bavaria, Germany)	04707516001
DQ ovalbumin	Molecular Probes, Invitrogen (Waltham, MA USA).	D12053
Total RNA isolation kit	Himedia India Pvt Ltd	MB602

Annexure III:

Cell culture reagents:

Name	**Source**	**Catalog**
RPMI-1640	HiMedia (Mumbai, India)	AL060A
Heat inactivated fetal bovine serum		RM9955-100ML
Penicillin-Streptomycin		P4333
DMEM		AL151A
Trypsin-EDTA solution		T3924

Annexure IV.

Dyes and Buffers:

Flow cytometry:

1. **Propidium Iodide (PI) Staining Solution:**

   50 µg/ml PI in 0.1 % Na-citrate + 0.1 % Triton X-100 in water,

   For 100 ml: Na Citrate 0.1 g+Triton X-100, 0.1 ml+ PI 5 mg+ Water 100ml

2. **Phosphate Buffer Saline (PBS):**

   0.8 % NaCl, 0.02% KCl, 2.7 mM KCl, 10 mM phosphate, pH 7.4

   For 100ml: NaCl: 0.8 g+ KCl: 0.02 g + Na_2HPO_4: 0.126 g+ NaH_2PO_4: 0.0451 g+ Distilled Water to make volume up to 100 ml.

3. **Cell fixation buffer:** 4% formaldehyde (100%) in PBS.

   For 100 ml: 10 ml of 40% formaldehyde in 90 ml of water.

4. **Cell permeabilization buffer:** 0.5% Tween-20 and 5% fetal bovine serum (FBS) in 1X PBS.

   For 100 ml: 0.5 ml Tween 20, 5 ml FBS and 94.5 ml PBS.

5. **CFSE dye stock (10 mM):**

   Dissolve 25 mg CFSE in 5.28 ml of DMSO, stored at -30^{0}C. Working concentration is 10 µM.

ELISA buffers and solutions:

6. **Assay Diluent:** 1X PBS + 10% FCS.
7. **Coating Buffer for ELISA:**

a. **0.1 M Sodium Carbonate:** 0.15 M sodium carbonate, 0.35 M sodium bicarbonate.

   Add $NaHCO_3$: 8.4 g, Na_2CO_3: 3.56 g and makeup the volume with distilled water to 1000 ml. adjust pH to 9.5.

b. **0.2 M Sodium Phosphate:** Add Na_2HPO_4: 12.49 g, NaH_2PO_4: 5.47 g; and makeup the volume with distilled water to 1000 ml, pH to 6.5)

8. **Wash Buffer**: 1X PBS + 0.05% Tween 20.
9. **Stop solution**: 0.2 M H_2SO_4.

Western blot:

10. **Cell lysis buffer**:

    0.5 M Tris Cl pH 6.8 - 2.5 ml (final concentration 50 mM).

    Glycerol - 2.0 ml.

    10% SDS - 4.0 ml.

    2-Mercaptoethanol - 1.0 ml

    0.1% Bromophenol blue - 0.5 ml

    Protease inhibitor cocktail (1X) and phosphatase inhibitor cocktail (1X).

11. **1.5M Tris Cl pH 8.8**: Dissolve 18.117 gm Tris base in D/W. Adjust pH to 8.8 with conc.HCl and make up final volume to 100 ml with D/W.
12. **0.5M Tris-Cl pH: 6.8:** Dissolve 6.057 gm Tris Cl in 100 ml.
13. **30% acrylamide mix:** 29 % acrylamide, 1 % N,N'-Methylene bisacrylamide.

    Acrylamide - 29.2 gm, Bisacrylamide - 0.8 gm and dissolve in D/W. Make up the final volume to 100 ml with D/W.

14. **10 % SDS:** 10 g of SDS in 100 ml of water.
15. **Tris-Glycine Electrophoresis Buffer:** 25 mM Tris base, 192 mM glycine (pH8.3) and 0.1 % SDS. For 10X (1 L), dissolve 30.0 g of Tris base, 144.0 g of glycine, and 10.0 g of SDS in 1000 ml of H_2O. Dilute to 1X with H_2O before use.
16. **Towbin transfer buffer**: 25 mM Tris, 192 mM Glycine, 0.1% SDS, 20 % Methanol. pH: 8.6.

CPRG assay:

17. **Lysis buffer** (25 mM Tris-phosphate, pH 7.8, 2 mM DTT, 2 mM CDTA, 10% glycerol, 0.5% Triton X-100).

    174 mg of Tris-phosphate, 3.08 mg DTT, 7 mg CDTA, 1 ml glycerol and 0.5 ml Triton X-100 all dissolved in 8.5 ml of water.
18. **CPRG buffer**: 100 mM 2-ME, 9 mM MgCl2, 0.125% NP-40 and 0.15 mM CPRG substrate in PBS.