ROLE OF NKR-P2/NKG2D AS TUMOR RECOGNITION AND ACTIVATION RECEPTOR ON DENDRITIC CELLS

THESIS

Submitted for the degree of

DOCTOR OF PHILOSOPHY

To

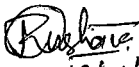JAWAHARLAL NEHRU UNIVERSITY

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2007

CERTIFICATE

The research work presented in this thesis has been carried out at the Centre for Cellular and Molecular Biology, Hyderabad. The work is original and has not been submitted in part or full for any other degree or diploma of any other university.

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Dedicated to

~ My Parents

Acknowledgements

It is an immense pleasure to express my gratitude towards my supervisor Dr. Ashok Khar for introducing me to this exciting area of research, encouragements and support during my research work. He had encouraged me to attempt thoughtful experiments and provided freedom to design and execute interesting experiments. His expert guidance, scholastic discussions helped me a lot to pursue this work. He had been a great mentor for me and I have learnt a lot from him.

I extend my profound regards to my co-supervisor Dr. S. Shivaji for his support in all the possible ways. I am also thankful to various members of S. Shivaji's group for their help at crucial time. It gives an immense pleasure to thank the director of CCMB Dr. Lalji Singh who encouragesand envisagesthe students to do quality science and for that he has established the best of the modern facilities and smooth ambiance at CCMB.

Mrs. Varalakshmi, Mr. Mubarak Ali, Mr. B.V.V Pardhasaradhi will be remembered who helped me directly in tissue culture facility. I specially thank Varalakshmi for help in generating monoclonals and keeping the reagents in the best forms. I also thank Mr. Mubarak for providing me various cell lines in very good conditions. I thank B.V.V. Pardhasaradhi for his help in transfections and providing reagents. It was pleasant to ask help from Johnson, Dayakar, Ramesh and late Chandramohan in tissue culture. It was more important that they fulfilled my request without delay. I thank former group members Leela, Zareena, Swamy and Srinath for their support in-group. I am thankful to Mr. Dwarkanath and Mr. Shekhar who helped me in handling animal experiments. I also thank Mrs. Nandini who helped me in the confocal microscopy.

I owe a great debt for Dr. Manjula for her help at very crucial time. I am indebted to Mrs. Hemalatha for her help in manuscripts preparation and submissions. The former lab members like Rajshekhar, Christine, Roshni, Savithri, Suvendu, and Sarvajeet made me familiar to various techniques in very short time after my joining in lab. I am thankful to all the previous lab members

and their support. I also thank Dr. Rana Anjum for providing antibodies. It was also enjoyable to be with all group members. South wing ground floor had pleasant atmosphere. Praveen, Sumit and Pallavi were excellent to interact. My friends like Satish, Ramparikshan, Ganesh, Gopal, Kaushlendra, Deepu and others were too good interact. It was also good to be in playground for cricket every morning with all friends at a time. It was wonderful to be with Ashish, I appreciate his trust in me and do remember the regular meal that we prepared for each other. Word fails to articulate my feeling toward Ashish, who helped me various ways. It was superb to interact with Dr. Sunit K. Singh and discuss various aspects of infection and immunology. I also thank my teachers Prof. S. Kundu and Prof. S.K. Srivastava for being in touch with me. I also thank my graduate and postgraduate teachers who taught me various subjects that encouraged me towards science. I specially thank my immunology teachers for teaching me immunology in a very exciting manner.

Words plunge insufficient to express my regards and emotions to my parents who inspired me for achieving higher education. I am indebted for their support and patience. I am thankful to my wife Richa and in-laws for their support, affection and sustained patience. I am thankful to my younger brother Amit for his affectionate and endless-support at very crucial time. I thank my cousin Ashish for his support. I also remember my cousins, uncles and aunts who helped me in all ways.

I would also acknowledge the help provided by various support groups, central facilities, instrumentation, animal house and photography at CCMB. I do appreciate the people of CCMB for maintaining an excellent place to pursue research.

I acknowledge the financial support of CSIR and CCMB.

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ABBREVIATIONS

Alkaline phosphatase (AP)

Ammonium chloride (NH$_4$Cl)

Ammonium hydroxide (NH$_4$OH)

Ammonium persulphate (APS)

Amino Acid (AA)

Antibody (Ab)

Antibody-dependent cellular cytotoxicity (ADCC)

Antigen/s (Ag/Ags)

Antigen-presenting cells (APC)

Base pairs (bp)

BCIP (5-bromo-4-chloro-3-indol phosphate)

B cell receptor (BCR)

Bone Marrow (BM)

Bone marrow derived DCs (BMDCs)

Bovine serum albumin (BSA)

c-type lectin like receptor (CLRs)

Cell adhesion molecules (CAMs)

Cluster of Differentiation (CD)

Complementary DNA strand (cDNA)

Complete medium (CM)

Counts per minutes (cpm)

Cytotoxic T lymphocytes (CTL)

4,6-diamidino-2-phenylindole (DAPI)

Dalton (Da)

Dendritic cells (DCs)

Deoxyribonucleotide (dNTPs)

Dimethyl sulfoxide (DMSO)

Dithiothreitol (DTT)

Dulbecco's modified Eagle's medium (DMEM)

Effector: target ratio (E: T)

Electrochemiluminescence (ECL)

Enzyme-linked immunofiltration assay (ELIFA)

Extracellular domain (ECD)

Ethylene diamino tetra acetic acid (EDTA)

Fetal calf serum (FCS)

Fluorescence activated cell sorter (FACS)

Fluorescein isothiocyanate (FITC)

Fragment crystallisable (Fc)

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

Glutathione S-Transferase (GST)

Granulocyte monocyte- colony stimulating factor (GM-CSF)

Haematopoitic stem cells (HSCs)

Heat shock proteins (HSPs)

Hour (h)

Immature DCs (iDCs)

Immature BMDCs (iBMDCs)

Immunoglobulin (Ig)

Immunoreceptor tyrosine-based activation motif (ITAM)

Immunoreceptor tyrosine based inhibitory motif (ITIM)

Iscove's modified Dulbecco's medium (IMDM)

Inducible NO synthase (iNOS)

Interferon (IFN)

Interleukin (IL)

Intraperitoneal (i.p.)

Killer cell immunoglobulin like receptor (KIRs)

Large granular lymphocyte (LGLs)

Lipopolysaccharide (LPS)

Lymph nodes (LNs)

Lymphotoxin (LT)

Macrophage (Mφ)

Major histocompatibility complex (MHC)

Mature dendritic cells (mDCs)

Mean fluorescence intensity (MFI)

Monoclonal antibodies (mAbs)

Monocytes (MOs)

MTT (3-4,5-dimethylthiozol-2-YL 2,5-diphenyltetrazolium bromide)

Natural cytotoxicity receptor (NCRs)

Natural Killer (NK)

Natural killer cell receptor (NKRs)

Natural killer group 2, member D (NKG2D)

Natural killer receptor protein-1 (NKR-P1)

Natural killer receptor protein-2 (NKR-P2)

Nitro-blue tetrazolium (NBT)

Nitric oxide (NO)

Nickel-nitrilotriacetic acid (Ni-NTA)

o-Phenylenediamine (OPD)

Phosphate-buffered saline (PBS)

Peritoneal DCs (PDCs)

Phenyl methyl sulfonyl fluoride (PMSF)

Plasmacytoid DCs (pDCs)

Propidium iodide reagent (PI)

Polymerase chain reaction (PCR)

Regulatory T cells (Treg)

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Seconds (Sec)

Sodium dodecyl sulphate (SDS)

Subcutaneous (s.c.)

T cell antigen receptor (TCR)

N,N,N',N'-tetramethylethylene diamine (TEMED)

Transforming growth factor (TGF)

T helper (Th)

Tumor infilitrating lymphocyte (TILs)

Tumor associated macrophage (TAMs)

Tumor necrosis factor (TNF)

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ABSTRACT

Tumor immunosurveillance is essential for the eradication of tumor or cancer from the host. DCs are sentinels and effectively respond to various 'danger signals' including tumor cells. DCs are professional antigen presenting cells and regulate anti-tumor immune responses by regulating activation and differentiation of effector T cells. Nevertheless, DCs also endow the activation of NK cells, B cells and other lymphocytes to participate in tumor immunosurveillance. This multitasking ability of DCs governs innate and adaptive anti-tumor responses.

In addition to their pivotal role in adaptive anti-tumor responses, the direct tumoricidal function of DCs against various tumor cells was recently confirmed. It was shown that DCs rapidly migrate in tumor mass and can induce tumor regression. For tumor cell recognition a 'tumor recognition mechanism' was thought to operate on tumoricidal DCs. In due course of time, it was revealed that DCs are equipped with multiple receptors for tumor cell recognition that includes various death inducing as well as NK cell activating receptors.

AK-5 tumor cell is a highly immunogenic rat histiocytoma of macrophage origin. Upon s.c transplantation AK-5 cells grow as a solid tumor and undergoes rapid rejection spontaneously, whereas i.p. transplantation leads to the development of ascites and the death of the host. Innate immune cells were found to be the major mediator of spontaneous rejection of AK-5 s.c tumor *in-vivo*, which is an important but a rare phenomenon. In addition it was observed that fixed AK-5 tumor cells could induce the activation of DCs confirming immunogenic surface molecule on AK-5 tumor cells.

Given the innate anti-tumor immune response of DCs, we have observed an imperative "NK cell-like mechanism" to be operating in DCs. DCs share a potent tumor recognition receptor "NKG2D/NKR-P2", along with NK cells and other lymphocytes to induce cytotoxicity. NK cell activation requires a balance of activation and inhibitory signals for their cytotoxic responses, which operate in accordance with 'missing self-hypothesis' for NKG2D mediated NK cell activation.

In this study we have shown that NKR-P2/NKG2D acts as a potent tumor recognition receptor on DCs, which endow tumoricidal potential of DCs. Being a distinct cell type in contrast to NK cells, we have also observed additional functions of NKR-P2/NKG2D on DCs. Work presented here insights the role of NKR-P2 on DCs, which could be an effective target for cancer immunotherapy.

Chapter 1 presents a brief introduction to DCs, origin and lineage of DCs, overview of DCs functional mechanisms, NK cell receptors, interaction of heat shock proteins with DCs and their involvement in anti-tumor immune responses.

Chapter 2 deals with the materials and methods used in carrying out this study.

Chapter 3 deals with the function of NKR-P2 in DCs mediated immune responses with the help of an agonistic anti-NKR-P2 mAb that activated DCs. In this study various functional consequences of NKR-P2 mediated DCs function were observed including DCs activation, NKR-P2 mediated tumoricidal effects of DCs, signal transduction involved in NKR-P2 mediated DCs activation, role of NKR-P2 in DCs maturation and also the therapeutic potential of anti-NKR-P2 mAb *in-vivo*.

Chapter 4 deals with the identification of a novel ligand irp94 (a 110-kDa heat shock protein) on AK-5 tumor cells for rat NKR-P2 on DCs and its functional role in inducing activation of DCs. In this study we have demonstrated expression of a 110-kDa heat shock protein on AK-5 tumor cell surface, which interacts with NKR-P2 on DCs to induce their activation.

In this study we have identified the role of NKR-P2/NKG2D on DCs mediated antitumor responses as well as interacting ligand(s) of NKR-P2/NKG2D on tumor cells.

CHAPTER I

Introduction

1.1 DENDRITIC CELLS - AN OVERVIEW

Dendritic cells (DCs) are a distinguished class of leukocytes and play a major role in antigen capture, processing and presentation. DCs were first discovered by Cohn and Steinman in 1973 (Steinman and Cohn, 1973; Steinman et al., 1974), though skin DCs were visualized by Paul Langerhans in 1868 (Langerhans, 1868; Valladeau, 2006). DCs, named for their dendritic shapes (from the greek 'dendron', meaning tree), are pivotal for both recognition of distinct Ags (antigens) and control of an array of responses (Steinman and Banchereau, 2007).

DCs are distributed at distinct anatomical locations (Banchereau and Steinman, 1998). As sentinel (Kubach et al., 2005), DCs maintain immunological homeostasis by sampling self-antigens (Hart, 1997; Steinman, 1991). Upon exposure to stimuli, immature DCs (iDCs) migrate to lymph nodes (LNs) and modulate the effector function of T cells by Ag presentation. DCs display 100 times better Ag presentation function than other Ag presenting cells (APCs) i.e B cells and macrophages (Mφ) (Steinman and Witmer, 1978). This superior efficiency of DCs categorizes it as a "professional Ag presenting cells".

Mφ function locally in resident tissue, whereas DCs engulf Ag, acquire mature phenotype, and migrate to local LNs. Monocytes (MOs) can be differentiated *in-vitro* to Mφ or DCs, but distinct gene expression profiles between Mφ and DCs are found, which confirm DCs as unique cell type (Lehtonen et al., 2007).

Although B and T cells are the mediators of immunity, they function under the control of DCs (Banchereau and Steinman, 1998). DCs regulate B-cell growth, thymus-dependent Ab responses (Inaba et al., 1983) and directly stimulate B cells to produce high amount of IgM, IgG or IgA (Dubois et al., 1997). DCs produce IL-1, IL-6, and TNF-α and help in the maturation of B cells (Akira et al., 1990). DCs are reported to promote IgG_1-restricted B cell responses *in-vitro* (Bajer et al., 2003). DCs produce CCL19, CCL20, and CCL21, that alone or in cooperation with chemokines, regulate B-cell migration in lymphoid organs (Dubois et al., 2001). Recently intra-vital imaging showed that, after exiting endothelial venules and before entry into LNs follicles, B cells survey DCs for further responses. Engagement of B cell receptor by DCs-associated Ag leads to calcium signaling, migration arrest, Ag acquisition and extrafollicular accumulation that confirm the role of Ag-specific B cell and DCs interactions in promoting T cell dependent Ab responses *in-vivo* (Qi et al., 2006).

Infected cells or tumors display low amounts of Ag (100 or less per cell) to specific T cells and lack co-stimulatory molecules, which are required for the clonal expansion of T

cells. DCs solve this critical problem by presenting large amount of MHC-peptides to T cells to harness effective immune response. DCs activate $CD8^+$ and $CD4^+$ T cells by presenting antigenic peptides through MHC I and MHC II, respectively (Banchereau and Steinman, 1998). High-resolution imaging showed that the 'probing motion' of DCs- processes enable DCs to contact ~5,000 T cells per hour (Lindquist et al., 2004).

DCs functions are sub-divided in to distinct subtypes, which display a unique heterogeneity in DCs population (Wu and Liu, 2007). Various evidences suggest that DCs adopt a unique division of labour through distinct subsets in-vivo. This distinction was recently confirmed by targeting one Ag to distinct subsets of DCs by chimeric mAb, and found that distinct subsets of DCs participate for Ag presentation in-vivo (Dudziak et al., 2007; Iwasaki, 2007). DCs heterogeneity is dependent on differences of origin, anatomical locations, cell-surface phenotype, and function; yet all DCs subsets display potent Ag presenting capacity for stimulating naive, memory, and effector T cells.

Less number of DCs in-vivo and lack of specific markers for isolation and characterization had impeded DCs research. The novel methods for DCs generation (Inaba et al., 1992), discovery of specific markers and reagents have accelerated DCs research, leading to characterization of an important cell type (DCs), which can be tamed to control various disorders including allergy, autoimmune disease, resistance to infection, immunodeficiency, and cancer. DCs have the unmatched potential for vaccination and cancer immunotherapy (del Hoyo et al., 2002). Although Ag-presentation function of DCs is well studied; direct cytotoxic function of DCs against various tumor cells is recognized now, which accomplish additional interest for DCs (Chapoval et al., 2000; Manna and Mohanakumar, 2002; Nicolas et al., 2007; Trinite et al., 2005). Figure 1.1 represents multiple aspects of DCs in antitumor immune responses.

1.1.1 Origin of DCs

DCs originate from hematopoietic stem cells (HSCs) in bone marrow (BM) (Blom and Spits, 2006; Metcalf, 2007). HSCs give rise to downstream population based on their bias towards lymphoid or myeloid precursors (Metcalf, 2007). Pathway biased population are divided in to two main progenitors i.e. common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs) (Liu et al., 2007; Metcalf, 2007; Shortman and Liu, 2002; Shortman and Naik, 2007; del Hoyo et al., 2002; Wu and Liu, 2007). Precursor transfer studies and limiting-dilution approach in mouse and human showed that both CLPs and CMPs can generate all types of DCs (Manz et al., 2001; Traver et al., 2000; Naik

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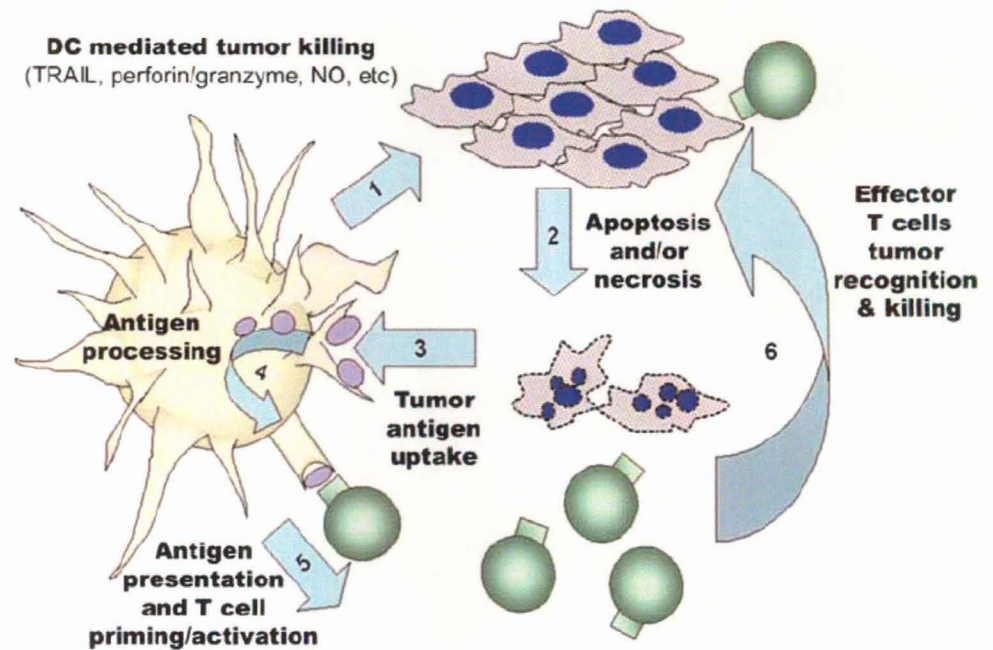
Figure 1.1: Model of DCs-mediated anti-tumor activity.

Following an encounter of DCs with tumor cells (1), tumor cells undergo death via apoptosis or necrosis (2). These dying tumor cells provide antigens for DC uptake, (3) which are then processed into peptides and loaded into MHC molecules (4). DC may then present epitopes from tumor antigen in the context of MHC I and MHC II molecules to prime and activate specific tumor-reactive T cells (5) leading to the development of effector T cells that can recognize tumors (6) causing tumor lysis and death (2), further diminishing the tumor burden (and contributing an additional pool of antigen for DC uptake)
Reference: (Wesa and Storkus, 2007)

et al., 2006). MOs are the precursors of DCs into the blood and LNs. Several findings support that DCs generation follows an extraordinary developmental flexibility (Leon et al., 2004).

DCs are found at various lymphoid and non-lymphoid locations but it is difficult to characterize lymphoid or myeloid DCs based on their anatomical locations. CLPs and CMPs population are segregated on the basis of the expression of Flt3 receptor (FMS-related tyrosine kinase3 receptor). Flt3 receptor is a receptor tyrosine kinase with homology to c-Kit (the receptor for stem cell factor) and c-fms (the receptor for macrophage colony stimulating factor). Flt3 receptor is highly expressed on hematopoietic progenitor cells (Lyman and Jacobsen, 1998). Despite of enigmatic heterogeneity of differentiated DCs, Flt3$^+$ CLPs and CMPs subgroup exhibit the decisive potential to generate majority of DCs (Karsunky et al., 2003).

Figure 1.2 shows the diagrammatic potential of Flt3$^+$ myeloid and lymphoid progenitors that generates majority of DCs. The importance of Flt3 receptor is also proved in DCs generation when Flt3$^-$ erythrocyte-magakaryocyte precursor produced DCs upon *flt3* gene transfection (Onai et al., 2006). Upon Flt3 ligand administration *in-vivo*, Flt3$^+$ progenitors and DCs progeny expands, whereas Flt3$^-$ downstream progenitors fail to generate DCs. Transplantation of CMPs and CLPs, and subsequent Flt3 ligand injection also increases DCs progeny of both precursor populations (Maraskovsky et al., 1996).

Amplification of Flt3$^+$CD11b$^+$ progenitor from mouse BM was achieved and progenitor cells develop into both CD11b$^+$ and CD11b$^-$ DCs, CD8α$^+$ and CD8α$^-$ DCs *in- vivo* (Hieronymus et al., 2005). Direct evidences of Flt3 receptor in the hematopoietic hierarchy suggests that Flt3 ligand can drive DCs development from both lymphoid and myeloid pathways with progenitors (Hieronymus et al., 2005 ; Leon et al., 2004). The fact that both the CMPs and CLPs generate majority of DCs of the spleen and thymus suggest that the phenotype of differentiated DCs can not reflects their lineage or origin (Karsunky et al., 2003; Metcalf, 2007).

1.1.2 Differentiation of DCs

CD34$^+$ stem cell can generate DCs in the presence of appropriate cytokines in BM or *in-vitro*. The concerted action of cytokines is proven to be the most crucial component for DCs differentiation (Zou and Tam, 2002). Under *in-vitro* conditions CD34$^+$ precursor cells, embryonic stem cells, cord blood cells, adherent and non-adherent PBMCs, MOs, embryonal carcinoma cells have been differentiated into DCs with the help of cytokines (Zou and Tam,
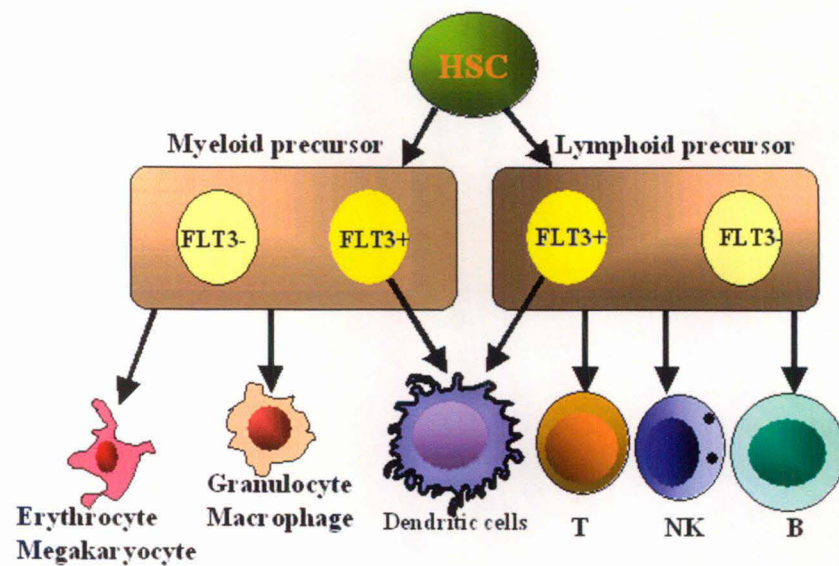
Figure 1.2: Developmental regulation of DCs:

The step towards the development of the types of blood cells is considered to be the formation of a series of precursor cells that have a strong bias towards either the myeloid or the lymphoid lineages. Originally, these were isolated as the CMPs and CLPs populations. Further work has defined a series of precursors of each type, rather than just two committed precursor forms. Of these, the myeloid precursors and the lymphoid precursors that express the Flt3 receptor possess the greatest capacity to form DCs. Reference: (Shortman and Naik, 2007).

2002). The prerequisite of DCs generation is to obtain the immature form of DCs, which may display all DCs function. GM-CSF (Granulocyte macrophage-colony stimulating factor) and FLT3 ligand are the most widely used cytokines for DCs generation from the precursor population.

GM-CSF: GM-CSF generates DCs from precursors, which display typical DCs function, specially maturation (Caux, 1996; Sallusto and Lanzavecchia, 1994). GM-CSF is the most preferable cytokine for DCs differentiation across species. IL-4 in conjunction with GM-CSF is also used in DCs production. GM-CSF/IL-4 can also convert Mϕs into DCs (Palucka et al., 1998). It was found that IL-4 is a preferable cytokine along with GM-CSF to suppress the development of monocytic progeny in DCs committed culture, since IL-4 downregulates CD14 monocytic marker (Lauener et al., 1990). However GM-CSF itself downregulates CD14 on MOs, hence only GM-CSF can be sufficient to generate DCs (Kruger et al., 1996). Surprisingly it was found that mice lacking GM-CSF receptor have normal amount of DCs, the reason was attributed to the fact that GM-CSF receptor shares subunit homology with IL-3 and IL-5 receptor, which may substitute for GM-CSF *in- vivo* (Vremec et al., 2000). It is recently proposed that GM-CSF generates 'inflammatory DCs' rather than steady state DCs (Shortman and Naik, 2007), because GM-CSF level rises during inflammation in the body (Cebon et al., 1994; Cheers, 1988).

FLT3 Ligand: FLT3 ligand produces steady state DCs and plasmacytoid DCs (pDCs) (Brasel et al., 2000b; Naik, 2005). In FLT3$^{-/-}$ mice less number of DCs were found (McKenna, 2000) and FLT3 ligand administration proliferates DCs *in-vivo* (Maraskovsky, 1996; Maraskovsky, 2000). FLT3 ligand in conjunction with GM-CSF has also been used for DCs generation (Brasel et al., 2000). FLT3 ligand induced DCs are CD24^{high} CD11b^{low} CD172a^{low} and CD24^{low} CD11b^{high} CD172a^{high} subsets, which correspond to the CD8$^+$ and CD8$^-$ DCs subtypes *in-vivo* (Naik, 2005). FLT3 ligand administration after hematopoietic cell transplantation also increases circulating DCs precursors (Chen et al., 2005). It was also reported that trancription factor STAT3 is required for FLT3 ligand dependent DCs differentiation (Laouar et al., 2003).

Other cytokines: IL-1β, IL-3, IL-7, IL-15, IL-21, TNF-α, SCF and other cytokines are also used in DCs generation *in-vitro* under specialized requirement. Although *in-vitro* generated DCs display many important DCs's functions yet these protocols do not produce identical DCs types constantly and might not be identical to DCs *in- vivo* (Shortman and Naik, 2007; Vuckovic et al., 2002).

1.1.3 DCs development and lineage complexity

Direct evidence for the myeloid origin of DCs was established upon transplantation of mouse CMPs into irradiated recipients that led to the reconstitution of the DCs in the spleen and thymus (Manz, 2001; Traver, 2000). Successful DCs generation from MHC II⁻ myeloid progenitors also suggests that DCs are myeloid in origin (Inaba, 1993). A $CX_3CR1^+CD117^+$ clonogenic progenitor that generates $M\phi$ and splenic DCs were identified in mouse BM (Fogg, 2006). The strong evidence for myeloid origin of DCs was established becuase MOs differentiate into DCs in the presence of GM-CSF and IL-4 in-vitro (Chapuis et al., 1997; Zhou and Tedder, 1996).

Thymic DCs and subpopulations of DCs in mouse spleen and LNs express markers associated with lymphoid cells, including CD2, CD4, CD8α, and CD25 (Vremec et al., 2000). Transfer of intrathymic lymphoid-restricted precursors into the thymus of irradiated recipients gave rise to both T cells and $CD8^+$ thymic DCs (Wu et al., 1995). This precursor showed the potential to produce $CD8^+$ and $CD8^-$ splenic DCs exclusively, when injected intravenously; and lacks the myeloid potential (Martinez del Hoyo, 2002; Wu, 2001). In addition mouse CLPs show the potential to differentiate into DCs both in- vitro and in-vivo (Manz, 2001).

Cells of the T-cell and B-cell lineages have immunoglobulin heavy-chain (IgH) gene D-J rearrangements. When found in DCs, such rearrangements display the lymphoid past. Around half of the DCs in the mouse thymus, but only a small number of the DCs in spleen and LNs, have such IgH D-J rearrangements (Corcoran, 2003). In humans $CD34^+Lin^-$ $CD45RA^+$ hematopoietic cell generates T, B, NK and DCs suggesting that developmental stages of DCs are more closely related to lymphoid lineage than myeloid lineage. It was reported that transcription factor RelB is essential for the development of myeloid-related $CD8\alpha^-$ DCs but not of lymphoid-related $CD8\alpha^+$ DCs (Wu et al., 1998). Thymic DCs and T cells develop simultaneously in the thymus from a common precursor population (Ardavin et al., 1993). Distinct roles for the NF-κB and c-Rel transcription factors are shown in the differentiation of DCs (O'Keeffe et al., 2005).

These findings indicated that there is a developmental flexibility in DCs lineage, and crucial events in the commitment to DCs lineage lies downstream of both CLPs and CMPs. Thus lineage-specific phenotypes or functions of 'myeloid' and 'lymphoid' DCs still remain elusive. With highly efficient xenotransplant model it was recently shown that even human DCs subsets possess indistinguishable expression patterns of surface phenotype and gene

transcripts regardless of their CMPs or CLPs origin (Ishikawa et al., 2007). Figure 1.3 shows the developmental complexity associated with the DCs lineage. The extent to which the precursors contribute to DCs development is yet to be completely explored (Dakic et al., 2004; Shortman and Liu, 2002; Dudziak et al., 2007; Schnurr et al., 2005). The pre-form of DCs i.e plasmacytoid DCs also show the lineage complexity, as they were found in IL-7R$\alpha^{-/-}$ mice, which are deficient in T and B cells suggesting their lymphoid-independent past, nevertheless they can be generated from myeloid precursors also (Yang et al., 2005a).

1.1.3.1 Role of transcription factors in lineage complexity of DCs?

Whether DCs development is commited to lymphoid or myleloid lineage has been moderately investigated based on the roles of transcription factors.

The role of Ikaros (a zinc finger DNA binding transcription factor) has been implicated in DCs haematopoiesis in mouse (Allman et al., 2006). The *Ikaros* gene is required for the development of all lymphoid lineages (Georgopoulos et al., 1994). Ikaros $^{-/-}$ mice show specific alterations in T cell development, severe alterations in B and NK cell development, and a significant reduction in DCs number (Wu et al., 1997). Ikaros dominant negative mice show more severe defects in lymphoid DCs. Selective defects in DCs manifested with the Ikaros null mutation suggest a tight linkage between development of T cells and CD8α^+ DCs. The complete lack of DCs in the lymphoid organs of Ikaros dominant negative mice also reflects an essential role for the 'Ikaros gene family' in the development of all DCs (Wu et al., 1997).

IK7 (A protein of Ikaros dominant negative product) aborts the interaction of IK7 with other proteins of Ikaros family. Overexpression of IK7 blocks DCs differentiation and differentially regulates the development of myeloid and lymphoid DCs. IK7 regulates the gene regulation by the modulation of Flt3 receptor mRNA downregulation in precursors. Culture conditions for myeloid and lymphoid DCs differentiation were defined for CD34$^+$Lin$^-$ CD10$^+$ precursors. IK7 only blocks the production of lymphoid related DCs suggesting a distinct signal control for DCs generation. Hence it was postulated that distinct molecular mechanism determines the diversity in DCs at the level of hematopoiesis (Wu et al., 1997).

RelB is a member of the NF-κB (Rel) family and is expressed in higher amounts in CD8$^-$ DCs compared to CD8$^+$ DCs in the spleen. Disruption of *relb* gene resulted in the defective development of DCs (Weih et al., 1995). Three-interferon regulatory factors (IRF), namely IRF-2, IRF-4, and IRF-8, also play important roles in the development of different DCs populations (Tamura et al., 2005). STAT3 is required for Flt3L-dependent steady state

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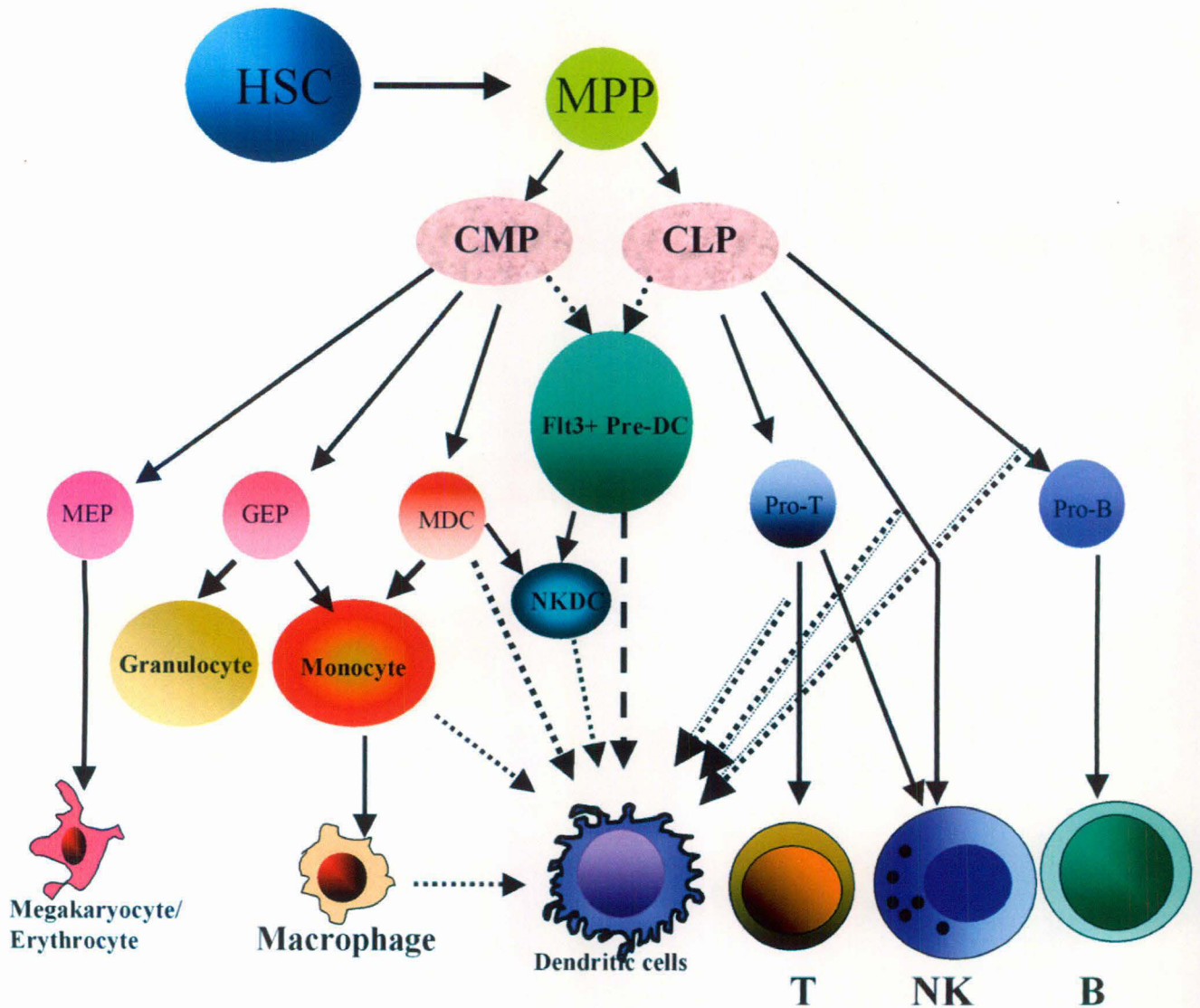
Figure 1.3: Differentiation of DCs.

The concept that DCs development proceeds through a sequence of strictly ordered and irreversible differentiation step is replaced by the concept of mixed pathways. Several evidences support that DCs developmental lineage is undistiguishable from the developmental pathways of other lymphocytes of lymphoid or myeloid past.
Reference:(Chen et al., 2007; Palucka et al., 1998; Wu and Liu, 2007).

DCs development. Deletion of *stat3* in hematopoietic cells abolished the effects of FLT3L on DCs development and led to a profound deficiency in the DCs compartment in lymphoid tissues. In contrast, deletion of *stat3* could not affect DCs development *in-vitro* in the presence of GM-CSF, indicating that STAT3 is not required for GM-CSF dependent inflammatory DCs differentiation (Bharadwaj and Agrawal, 2007; Laouar et al., 2003).

1.1.4 Heterogeneity of DCs

Since the discovery of DCs, it was envisaged that there are many distinct subtypes of DCs (Steinman et al., 1974). Differences in location, life cycle and intrinsic abilities of Ag presentation enable each DCs subset to have distinct roles in immunity and in the maintenance of tolerance. The interactions among DCs subsets allow the integration of the intrinsic abilities of different DCs to enhance the ability of the 'DCs network' against multiple infections (Villadangos and Schnorrer, 2007). Each subtype is of great importance since they may modulate specialized immune responses in distinct environment.

Heterogeneity in DCs subtype was strongly supported by *in-vitro* culture milieu, since different culture conditions generated different DCs subtypes from DCs-precursors (Chung et al., 2007; Dakic and Wu, 2003). The development of different subsets of DCs and their interrelationship is poorly understood (Szeberenyi et al., 2000). Distinct surface Ags that may characterize DCs have only become known recently. DCs are heterogeneous, and based on developmental pathway, location, migratory properties and detailed immunological functions their subsets are categorized as.

1.1.4.1 Pre-Dendritic cells (Pre-DCs): Pre-DCs are cells without DCs form or function but develop into DCs with little or no division. Different types of pre-DCs give rise to different DCs subtype with or without variable stimuli. Example of Pre-DCs is a precursor of steady state DCs i.e plasmacytoid DCs (pDCs). pDCs, so named because of cytologic similarities to Ab-producing plasma cells. pDCs are recently identified, round, 'non-dendritic' cells that produce large amount of typeI IFNs by variable stimuli (Colonna et al., 2002). Under inflammatory response pDCs can also be differentiated into mDCs (mature DCs). pDCs circulate through the blood and lymphoid tissues and only acquire the typical DCs morphology after activation, which is accompanied by the release of IFNs. The role of pDCs in Ag presentation and T cell priming is unclear, hence sometimes referred as 'IFN-producing cells' (Colonna et al., 2002; Colonna et al., 2004; Liu, 2005).

1.1.4.2 Steady state DCs: These DCs exhibit typical dendritic form and function in steady state. Based on the migratory properties these conventional DCs are further divided into two groups namely Migratory DCs and Residents DCs.

1.1.4.2.1 Migratory DCs: Migratory DCs have an immature phenotype in the peripheral tissues, where they survey environment and engulf extracellular material (Valladeau and Saeland, 2005). These DCs migrate through the afferent lymphatics to the local LNs and acquire a mature phenotype. The migration and maturation of these DCs occurs even in germ-free animals. These DCs serve as major sentinel in peripheral tissue and migrate to LNs in response to 'DANGER SIGNAL' (Mathers and Larregina, 2006; Wu and Liu, 2007). Migratory DCs display a mature phenotype only upon reaching to LNs. Similar migration to LNs also occurs at slow rate under normal physiological conditions. Examples for such DCs are langerhans cells (Valladeau, 2006) of epidermis, dermal DCs, interstitial DCs of epidermis, intestinal epithelium, respiratory and reproductive tract (Tacke and Randolph, 2006). Migratory DCs, which develop from precursors in peripheral tissues and travel through the afferent lymphatics to reach the local draining LNs, constitute ~50% of all LNs DCs (Randolph et al., 2005a). There are two subtypes of migratory DCs. The first subtype is found in all LNs, which corresponds to the interstitial DCs. The interstitial DCs contained in subcutaneous LNs migrate from the dermis and are often termed dermal DCs. Subcutaneous LNs also contain a second population of migratory DCs, namely the Langerhans cells, which migrate from the skin epidermis to LNs (Randolph et al., 2005b; Sallusto, 2001). The paucity of DCs in the efferent lymph has led to the idea that DCs die in the LNs (Randolph et al., 2005a), but new reports suggest that migratory DCs may traffic further to the BM (Cavanagh, 2005), thymus (Bonasio, 2006) and peritoneum (Mitra et al., 2004).

1.1.4.2.2 Resident DCs: Resident DCs reside in LNs i.e primary and secondary lymphoid organs (Steinman et al., 1997). Lymphoid-organ DCs are the blood-derived or resident DCs, which constitute the LNs DCs, splenic and thymic DCs. They can be subdivided into three types that are categorized by the expression of CD4 and CD8: $CD4^+$ DCs, $CD8^+$DCs and $CD4^-CD8^-$ DCs (Villadangos and Heath, 2005). Lymphoid-tissue resident DCs capture and present Ags in lymphoid tissue. These DCs display an immature phenotype and are proficient in Ags processing and presentation. Lymphoid resident DCs develop from BM- precursors within the lymphoid organs without previously trafficking through peripheral tissues (Kabashima, 2005; Naik, 2006). Furthermore, in the absence of infection, the resident DCs

maintain an immature phenotype throughout their lifespan (Wilson, 2003). They can be distinguished from migratory DCs in the LNs by their low cell-surface expression of MHC II and T cell co-stimulatory molecules (Wilson, 2003;Henri, 2001). Therefore, almost all splenic DCs and approximately half of the LNs-DCs are immature under steady state conditions. In rats two distinct subsets of spleen DCs exhibit different cytokine production and T cell stimulatory activity (Voisine et al., 2002). In mouse, these lymphoid DCs are further segregated into $CD8\alpha^+$ and $CD8\alpha^-$ DCs. These $CD8^{+/-}$ DCs differs in thieir immunological function (Villadangos and Heath, 2005).

1.1.4.3 Inflammatory DCs: A specialized class of DCs, which was found under inflammatory conditions *in-vivo*, is recently termed 'inflammatory DCs', since they are not found under steady state condition. Infection with influenza virus to pDCs produced inflammatory DCs. Similarly infection with *Listeria monocytogens* generates TipDCs (TNF and iNOS producing DCs) (Serbina et al., 2003; Tacke and Randolph, 2006). Since *de-novo* generation of specific cell type is surprising, the TLRs (Toll like receptor) for microbial products are attributed for inflammatory DCs generation that appears on early stage of hematopoiesis and their ligation can deflect the normal pathways of DCs differentiation (Krutzik, 2005). The contribution of human MOs-derived DCs to immune responses becomes evident during inflammation and also support the *de-novo* generation of inflammatory DCs (Le Borgne, 2006; Randolph et al., 1999).

1.1.5 Role of DCs in immune tolerance

The ability of immune system to distinguish between self and non-self and between innocuous and harmful foreign agents is controlled by the mechanism of tolerance. Tolerance is the specific inability of host to respond to self-Ags without the need for immunosuppressive drugs, which is generated centrally and peripherally (Woltman and van Kooten, 2003).

Central tolerance involves deletion of self-reactive T cells upon interaction with DCs in thymus whereas peripheral tolerances include the induction of cell death or the development of a state of unresponsiveness (anergy) of T cells (Morelli and Thomson, 2003). Exposure of newborn animals to foreign Ags often results in immunological tolerance to those Ags. This phenomenon is known as neonatal tolerance (Billingham et al., 1953). Re-examination of the classical neonatal tolerance experiments revealed that the intrinsic tolerance of the neonatal immune system is dependent on DCs function (Ridge et al., 1996),

which also maintains the immune tolerence in adults (Chen et al., 2006; Morelli and Thomson, 2007; Steinman et al., 2003).

Presence of self-Ags and control of maturation of DCs is the fundamental factor in inducing peripheral tolerance (Lange et al., 2007; Usharauli, 2005). DCs are highly interactive cells with extensive membrane processes that facilitate cell clustering and interaction with other cells, including T and B cells. DCs also exhibit a remarkable capacity to acquire plasmamembrane and intracellular proteins from other live APC and lymphocytes, and these acquired Ags can be cross-presented to MHC I-restricted CTLs (Harshyne et al., 2001).

The immature or mature stage of DCs alters the DCs function bimodally (Shakhar et al., 2005). According to theories such as 'The danger model' (Gallucci and Matzinger, 2001; Matzinger, 2002) and 'Infectious–nonself model' (Medzhitov and Janeway, 2002), in the absence of inflammation DCs remain in a quiescent immature stage and only deliver signal to TCR without co-stimulation and tolerize self-reactive peripheral T cells. Ags presentation by steady-state DCs establishes T cell tolerance to prevent adverse reactions to self when the same Ags are presented upon further immunization (Dudziak et al., 2007; Steinman et al., 2003). However the reports of different T cell responses, and distinct role of different subset of DCs in tolerance, indicates a more complex regulation of tolerance (Liu, 2001).

Tolerogenic DCs include immature, maturation-resistant or alternatively activated DCs that express low surface MHC I and MHC II , have a low co-stimulatory to inhibitory signal ratio and have an impaired ability to synthesize Th1 driving cytokines (such as IL12p70). Several observations favor the idea that local microenvironment determines the tolerance function of DCs, For example the function of DCs at immune privileged sites such as eye is tolerogenic rather than immunogenic (Streilein, 1999), thus non-inflammatory responses could be explained as a part of the naturally skewed DCs-dependent T cell response at different anatomical locations.

Although inflammatory signal induces maturation of DCs yet maturation stimuli *per se* do not always induce immunogenicity. Some maturation stimuli upregulate MHC II and co-stimulatory markers but fail to secrete Th1 polarizing cytokines. This in turn induces tolerogenic DCs, which is also called 'semi-mature DCs' (Gad et al., 2003). Figure 1.4 summarizes the status of DCs that are important in the induction of T cell anergy or immunity. In an effort to generate tolerogenic DCs, *in-vitro* propagated DCs have been manipulated through exposure to various anti-inflammatory and immunosuppressive agents. These agents target DCs differentiation and function by various mechanisms (Hackstein and
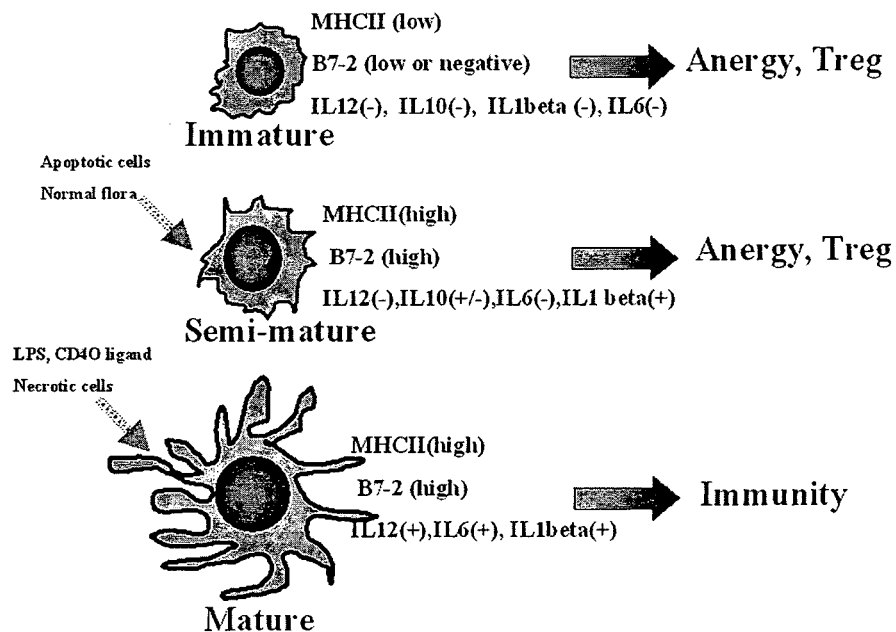
Figure 1.4: DCs maturation status governs tolerance and immunity.

The involvement of DCs in tolerance is more complex than earlier envisaged. Immature DCs can induce T cell anergy by their low expression of MHC II and B7-2 and lack of release of pro-inflammatory cytokine. It is clear now that high MHC class II and CD86 are not sufficient to induce T cell immunity. A new status depndent subset of DCs 'semimature DCs',which is tolerogenic cannot be distinguished from mature DCs by their surface markers. They express high MHC II levels and B7-2. In the absence of microbial stimulation 'semimature DCs' do not produce pro-inflammatory cytokines.'Semimature DCs' are induced by several factors including apoptotic cells or the gut bacteria.

T cell activation requires fully mature DCs, which shall have high MHC II, high B7-2 and can also release pro-inflammatory cytokines such as IL-12, IL-6, TNF-α and IL-1β. Conversion of mature DCs can be triggered by CD40L, LPS, CpG via their receptors. The induction of anergy can also be influenced by non-inflammatory cytokines such as IL-10 and TGF-β.

Reference: (Gad et al., 2003).

Thomson, 2004) and include IL-10, TGF-β1, inducers of cyclic-AMP such as prostaglandin E$_2$, histamine, neuropeptides, vitamin D$_3$ metabolite and its analogues, glucosamine, antioxidant *N*-acetyl-L-cysteine, ligands for inhibitory immunoglobulin-like transcript receptors (such as the MHC Ib molecule HLA-G), and cobalt protoporphyrin (to induce haem oxygenase-1 expression (Akiba et al., 2000; Kalinski et al., 1999; Morelli and Thomson, 2007;Delespesse et al., 1999; King et al., 1998). All these molecules prevent DCs maturation and/or activation or impair the capacity of DCs to produce bioactive IL-12p70. In addition, some of these agents prevent the nuclear translocation of NF-κB family members, which are required for the differentiation of DCs (Chauveau, 2005; Hackstein and Thomson, 2004; Morelli and Thomson, 2003).

The induction of T cell anergy by DCs involves incomplete maturation of DCs, blockade of B7 family co-stimulatory receptors, or the influence of specific non-inflammatory cytokines, such as IL-10 or TGF-β. In contrast to the 'semimature' tolerogenic DCs (Lutz and Schuler, 2002), blockade of MHC II expression and several co-stimulatory and adhesion molecules are found in tolerogenic DCs. Additionally, it has been reported that supernatants from immature IL-10 treated human DCs release reduced amounts of inflammatory cytokines such as IL-1β, IL-6 and TNF-α that suppress T cell function (Brossart et al., 2000; Faulkner et al., 2000; Steinbrink et al., 1999).

Active suppression by Treg (regulatory T cells) also plays a pivotal role in peripheral tolerance (Mahnke and Enk, 2005). CD4$^+$ CD25$^+$ Treg cells control Th1 type responses to foreign Ags *in-vivo* (Oldenhove et al., 2003). DCs induce the expansion of functional CD25$^+$ CD4$^+$ Treg cells (Yamazaki et al., 2003). Treg cells are CD25$^+$, CD4$^+$ T cells that fail to secrete IL-2 upon TCR stimulation and exhibit anergic phenotype. Treg block the function of other effector CD4$^+$ and CD8$^+$ T cells to achieve immune tolerance (Steinman et al., 2003). Treg include the variety of T cells that display regulatory function *in-vivo* and *in-vitro* (Kang and Datta, 2006). DCs also control peripheral tolerance by inducing the differentiation of Treg cells. Activation state of DCs and local cytokines play imperative role in the differentiation of Treg cells (Rutella et al., 2006).

Treg cells may be induced by iDCs or by semimature DCs. However once activated Treg cells suppress responses of both CD4$^+$ and CD8$^+$ T cells via an Ag non-specific mechanism (Lange et al., 2007). A subset of CD8$^+$ CD28$^-$ Treg cell induces upregulation of inhibitory receptors in DCs. These inhibitory receptor includes ILT3 and ILT4, which is responsible for DCs induced hypo-responsiveness of alloreactive CD4$^+$ T cells (Mahnke et

al., 2007a). However, the mechanisms by which DCs induce Treg cells and vice-versa are the intense area of research currently.

CCR7-mediated migration of naive T cells into the secondary lymphoid organs is a prerequisite for their encounter with mDCs. Recently it was shown that CCR7 is required for the *in-vivo* function of CD4$^+$ CD25$^+$ regulatory T cells (Schneider et al., 2007). DCs acquire a semi-mature phenotype and LNs homing potential through interaction with CD4$^+$CD25$^+$ regulatory T cells, which is critical for inducing peripheral tolerance (Bayry et al., 2007). Immunosuppressive functions of DCs by CD4$^+$ CD25$^+$ Treg cells have been shown by B7-H3 receptor *in-vivo* (Mahnke et al., 2007b). Tolerogenic semimature DCs suppress experimental autoimmune thyroiditis by activation of thyroglobulin-specific CD4$^+$CD25$^+$ T cells (Verginis et al., 2005). DCs directly control the function of Foxp3+ CD25$^+$CD4$^+$ Treg cells (Fehervari and Sakaguchi, 2004). Transplants tolerated through a process known as 'Infectious tolerance' evoke continuous recruitment of Treg cells that are necessary to maintain the unresponsive state. This state is maintained for a long-term and requires continuous Ag exposure. Using mice deficient in Treg cells, it has been shown that quiescent donor DCs laden with histocompatibility Ag can induce Treg cells *de novo* to mediate transplantation tolerance, whereas fully activated DCs fail to perform similar function (Yates et al., 2007). In human, IDO (indoleamine 2,3-dioxygenase) mediated T cell suppressor activity was found in mature and immature CD123$^+$ DCs. IDO$^+$ DCs was also found *in-vivo*, which display the unique subset of DCs with suppressive T cell function (Munn et al., 2002).

Given the importance of Treg cells and their dependence on DCs, potential therapeutic strategies against transplantation and autoimmune disorders can be pursued by taming DCs status, which is an important area of research currently (Rutella and Lemoli, 2004; Xiao et al., 2006).

1.1.6 DCs Response: "Danger model" Vs "Self-Nonself discrimination"

Self-nonself discrimination 'SNSD' model (Leng and Bentwich, 2002) suggests that immune response is generated whenever body encounters foreign substance. 'Danger model' (Matzinger, 2002) does not assume that discrimination between self and nonself is sufficient for immune system. Although both models agree that there is a need for crucial discrimination at the effector stage of immune response. 'Danger model' instead suggests that an evolutionary useful system (immune system) should contemplate on dangerous things, rather than only foreign entities.

In various aspects 'danger model' supersedes 'SNSD model' by including a wide range of immune responses. 'Danger model' allows a flexible immune system that also recognizes 'changing-self' (Matzinger, 2002).

Both the models are based on the principle that the presence or absence of second signal determines immune responsiveness or tolerance by APCs (Matzinger, 2002). Although both models differ greatly in detail, they assume that resting APCs can be activated by signals from their immediate environment. The 'self-nonself model' has found support with the discovery of the evolutionarily conserved Toll like receptors (TLRs), which act as pattern recognition receptors for the components of bacteria and fungi, and initiate immune responses (Aderem and Ulevitch, 2000; Medzhitov and Janeway, 2000; Medzhitov et al., 1997).

1.1.6.1 DCs responses to 'DANGER SIGNAL'

According to 'Danger model' DCs can also respond to substances that can originate because of cellular damage, along with non-self molecules. Hence 'danger signal' consists of agents produced by cells undergoing stress or abnormal cell death, which can be perceived by DCs to induce activation and maturation.

DCs are activated by 'danger signals' from injured cells, which can happen upon exposure to pathogens, toxins, and mechanical damage. Many danger signals have been shown to be constitutive or inducible, intracellular or secreted, or even a part of the extracellular matrix (Cvetanovic et al., 2006).

Cells that die by normal programmed cell death processes are usually scavenged before they disintegrate, whereas cells that die necrotically release their contents, hence any intracellular product could potentially be a danger signal when released. Danger signals also include any substance made, or modified, by distressed or injured cells. Ideally, danger signals should not be sent by any healthy cells or by cells undergoing normal death (Gallucci and Matzinger, 2001), failing to that may lead to autoimmunity. Various endogenous and exogenous signals act as the mediators of danger signal that induces DCs activation and maturation. DCs maturation is a crucial phenomenon that greatly regulates immune responses.

1.1.6.2 DCs Maturation

DCs are conveniently categorized as "immature" and "mature" forms, which discriminate between two well-characterized phenotypes of DCs. The ability of DCs to

regulate immunity is dependent on their transition from 'immature' to 'mature' phenotype. Thus term "maturation" refers to an intricate differentiation process whereby DCs respond to an environmental stimulus and regulate adaptive immunity (Munz et al., 2005b).

DCs degrade endogenous Ags by intracellular proteases and peptidases to provide peptides in the cytosol. These peptides, bind to TAP (transporter) gene products and are delivered to the MHC I compartment to be incorporated in the nascent peptide binding groove generated by folding of the MHC I before moving to the cell surface. This does not occur in the presence of proteasome or Golgi transport inhibitors and defines a conventional pathway. In DCs, several additional mechanisms also operate, which allow the presentation of exogenous Ags by MHC I (Hart, 1997; Lanzavecchia, 1996). DCs respond to multiple Ags (endogenous or exogenous; Table 1.1 (O'Neill et al., 2004)) and present them to T cells with MHC I and   MHC II.   Both MHC I and MHC II pathways of Ag presentation are differentially regulated during DCs maturation (Delamarre et al., 2003).

Typically exogenous Ags are presented through MHC II and endogenous Ags are presented through MHC I. However DCs can also present the exogenous Ags by MHC I, a phenomenon called as "Cross presentation" (Rock, 2006). During cross-presentation, non-replicating protein Ags are internalized and somehow gain access to the cytoplasm before being processed by the proteasome for peptide presentation on MHC I. Critical steps seem to occur from less-acidic compartments. Cross-presentation allows DCs to induce CD8$^+$ T cell responses to immune complexes, non-replicating forms of microbes and vaccines, and dying cells (Steinman and Banchereau, 2007). MHC II exhibit a specialized organization on the DCs's plasma membrane in contrast to other APCs. It was recently demonstrated that tetraspanin CD9 mediated lateral association of MHC II is found on surface of DCs only. This facilitates the formation of MHC II and TCR-complex during Ag presentation (Unternaehrer et al., 2007). mDCs are skilled in stimulating naive T cells and also control the quality of T cell responses. mDCs can also boost functionally superior CD8$^+$ T cell without foreign epitope (Dhodapkar et al., 2000).

1.1.6.2.1 Mechanism of DCs maturation

In peripheral tissue DCs exist in immature form, with very high endocytic activity to engulf small and large solutes by macropinocytosis, phagocytosis and clathrin-mediated

Classes	Stimuli	Receptors
TLR-Agonists	CpG DNA, HSV DNA	(TLR9)
	R-848, ssRNA	(TLR7,8)
	Imiquimod	(TLR7)
	Flagellin	(TLR5)
	Bacterial lipopeptides	(TLR1)
	Peptidoglycans,	(TLR2)
	Lipoproteins,	(TLR2)
	Glycolipids,	(TLR2)
	Hsp70	(TLR2)
	dsRNA, polyl:C	(TLR3)
	LPS	(TLR4)
	Hsp60, Hsp70,	(TLR4)
	Hyaluronan,	(TLR4)
	β-defensins	(TLR4)
	Flagellin	(TLR5)
TNF family-Molecules	TNFα	TNFR
	CD40 Ligand	CD40
	Fas Ligand	CD95
	TRANCE	RANK
	TNFSF14	TNFRSF14
Cytokines	IL1β	IL-1R
	IL6	IL-6R
Growth factors	TSLP	IL-7Rα/TSLPR
Interferons	IFNα	IFNAR1
Adhesion molecules	Agonistic mAb	CD66a
Costimulatory molecules	Agonistic mAb	B7-DC
Immune-complexes	Opsonized Ags	Fc receptors
Microbes	Viruses Bacteria	
Activated lymphocytes	CD4$^+$, CD8$^+$ T cells NK cells, NKT cells, Vδ1$^+$$\gamma\delta$ T cells	
Others	Uric acid	
Tumor Cells	Necrotic cells Apoptotic cells Fixed tumor cells (Alli and Khar, 2004)	

Table 1.1. List of agents and their receptors, which induce DCs maturation.
Reference: (O'Neill et al., 2004).

endocytosis (de Baey and Lanzavecchia, 2000). DCs have an array of receptors for Ag uptake, that includes c-type lectin like receptors, DC-SIGN, MMR (MRC1, DEC-205, BDCA-2, Langerin, Dectin-1; FcRs FcγRI (CD32), FcγRII (CD64); integrins αVβ5, CD11b/CD18, CR3, CD11c/CD18, CR4; scavanger receptor CD36, LOX-1, CD91; and aquaporins for fluid materials. Many of the Ag uptake receptors also participate in signaling or cell-cell interactions (O'Neill et al., 2004). iDCs synthesize large amounts of MHC II intracellularly, however only low surface level of MHC I and MHC II were found since $\alpha\beta$-dimers of MHCs are targeted to late endolysosomal compartment (also referred as MHC II compartment). After exposure to maturation stimuli, endocytosis increases transiently but downregulates later on, which is regulated by Rho family GTPases (Garrett et al., 2000). Maturation inducing agents stimulates DCs to extend actinin tubules from endolysosomal compartment to the plasmamembrane (Chow et al., 2002).

A key mechanism responsible for DCs maturation is the activation of lysosomal function, which slowly degrades the internalized Ags and facilitates efficient loading of peptides. Maturation stimuli activates vacuolar H^+ pump, which enhances lysosomal acidification, Ag proteolysis and facilitate the formation of peptide-MHCII complex (Trombetta et al., 2003). It was reported that differential lysosomal proteolysis in DCs also determines the fate of internalised-Ags (Delamarre et al., 2005).

In iDCs MHC II-β-chain cytoplasmic tail remains ubiquitinated and required for the sequestration of MHC II in multivesicular bodies, which is essential for endocytosis (Chow and Mellman, 2005). A transient aggregation of ubiquitinated proteins was found during DCs maturation (Lelouard et al., 2002). Notably, ubiquitination of MHC II stops upon maturation, resulting in the accumulation of MHC II on surface. DCs thus exhibit a unique ability to regulate MHC II surface expression by selectively controlling MHC II ubiquitination (Shin et al., 2006). During DCs maturation, surface MHC II level can increase from ~5 to 20 fold, while CD86 level can increase up to ~100 fold. The upregulation of surface MHC II reflects post-translational events. MHC II transcripts increase only slightly upon maturation hence the major change is attributed to the transport of MHC II during DCs maturation (Cella et al., 1997; Pierre and Mellman, 1998). Upon full maturation, DCs become specialized for carrying a particular Ags and cannot capture other foreign Ags (Banchereau and Steinman, 1998; Pierre et al., 1997).

Thus the conversion of 'immature' to 'mature' DCs is accompanied by a marked cellular reorganization, including the redistribution of MHC II from endolysosomal compartment to plasmamembrane, and downregulation of endocytosis, which was attributed

to slow-down the clearance of MHC II from surface (Pierre and Mellman, 1998). Lipid and glycolipid Ags expressed on pathogens or self tissues are presented by DCs to T cells on CD1 molecules (CD1a-d), which are structurally similar to MHC I but are specialized to bind lipids instead of peptides (Joyce and Van Kaer, 2003; Moody and Porcelli, 2003). Processing of lipid Ags onto CD1 molecules is carried out in specialized intracellular compartments, much like Ags processing onto MHC II. CD1a present lipid Ags to a variety of lymphocytes, including T cells with substantial T-cell receptor diversity as well as natural killer T (NKT) cells. These highly sophisticated processes also refer DCs as 'Ag-presenting machines' (Mellman and Steinman, 2001).

During maturation, expression of adhesion molecule increases, and DCs extend long dendritic process (membrane folds) that helps in their T cell interaction capabilities. DCs maturation takes place concomitantly with the migration of the DCs from peripheral tissues to LNs where they arrive through the lymphatic vessels (Steinman et al., 1999).

DCs mobilize within the extracellular matrix and migrate from peripheral sites to lymphoid organs upon maturation. Surface-proteoglycan expression changes quickly upon exposure to stimuli of maturation and play a major role in DCs interaction with extracellular matrix. Upregulated proteoglycans also associate with actinin (cytoskeletal component) for the enhanced mobility of DCs during maturation (Averbeck et al., 2007).

DCs also reestablish their chemokine receptors, which modulate their migratory properties. DCs maturation also results in increased expression of adhesion and co-stimulatory receptors involved in the formation of the immunological synapse and induces DCs to secrete cytokines (IL-1β, IL-10, IL-12), which are critical in determining the Th1 or Th2 type of responses (Steinman et al., 1999).

Another important effect of DCs maturation is the induced secretion of chemokines that recruit MOs, DCs, and specific subsets of T cells into the local environment. Thus, maturation conveys iDCs the ability to migrate from the tissues to T cell zones of LNs, which is mediated by the differential regulation of DCs chemokine receptors such as CCR1, CCR5, and CCR7 (Cravens et al., 2007; Heinzel et al., 2007; Sanchez-Sanchez et al., 2006; Trombetta and Mellman, 2005). It was recently reported that mDCs move faster than iDCs before dispersion in T cell zone, after dispersion mDCs stop migration and join DCs network in the LNs, which is essential for efficient Ag presentation *in-vivo* (Dudziak et al., 2007).

Activated NK cells induce DCs maturation *in-vitro* and *in-vivo* (Mocikat et al., 2003; Fujii et al., 2002; Hermans et al., 2003). NK, NKT, and γδT cells induce DCs maturation by a

combination of cytokines and cell contact dependent manner (Leslie et al., 2002; Gerosa et al., 2002; Fernandez et al., 1999; Fujii et al., 2004).

Thus, DCs maturation is a highly controlled phenomenon that is generated by distinct stimuli and controlled by a sophisticated mechanism, which play a pivotal role in DCs mediated immunity (Steinman et al., 1999). Figure 1.5 shows the phenotypic distinction associated with DCs maturation.

1.2 DCs-NK INTERACTIONS: BI-DIRECTIONAL CROSS TALK

DCs and NK cells are the essential components of innate immune system; their interaction controls the initiation and regulation of adaptive immune responses (Fernandez et al., 2002; Walzer et al., 2005a; Xu et al., 2007; Zitvogel, 2002; Zitvogel et al., 2006). NK cells show cytolytic function against tumor and virus infected cells, and DCs are equipped for Ag presentation function (Moretta, 2002). NK cell functions can be strongly enhanced by IL-12 produced by the activated DCs (Granucci et al., 2006). IL-12 produced by DCs activates NK cells, which confer systemic antitumor immunity in-$vivo$ (Gerosa et al., 2002; Granucci et al., 2004; Miller et al., 2003). Interaction between DCs and NK cells is crucial for the activation of effective antiviral immunity (Andoniou et al., 2005; Della Chiesa et al., 2005). By using selective depletion of DCs in mouse, it was recently shown that NK cells could not acquire its effector function without priming by DCs (Lucas et al., 2007). A unique NK cell subset ($CD3^-CD56^+$) triggers MOs to differentiate into immunopotent DCs in LNs in chronically inflamed condition; this differentiation of MOs to DCs requires direct contact of MOs with NK cells and is mediated by GM-CSF and CD154 derived from NK cells (Zhang et al., 2007a). Functional interactions between DCs and NK cells also result in their mutual regulation. For example: $trans$-presentation of IL-15 by IL-15Rα on DCs stimulates the cytotoxic activity of NK cells and their ability to produce IFN-γ (Degli-Esposti and Smyth, 2005; Koka et al., 2004).

Upon inflammatory stimuli, DCs produce IL-12, which is an inducer of IFN-γ production by NK cells. Upon virus-induced tissue damage, IL-12 dependent NK-DCs cooperation establishes the rapid initiation of IFN-γ based antiviral immune responses (Walzer et al., 2005b). Similarly, Ags exposure to DCs produces several chemokines (CCL3, CXCL8), which recruits NK cells having the receptors of CCL3 and CXCL8 to exhibit cytotoxicity (Munz et al., 2005b). Human blood-DCs also enhance the cytotoxic potential of NK cells upon IFN-γ activation; however cellular contact between activated DCs and NK cells is a prerequisite for enhancing NK cell function (Moretta et al., 2007).
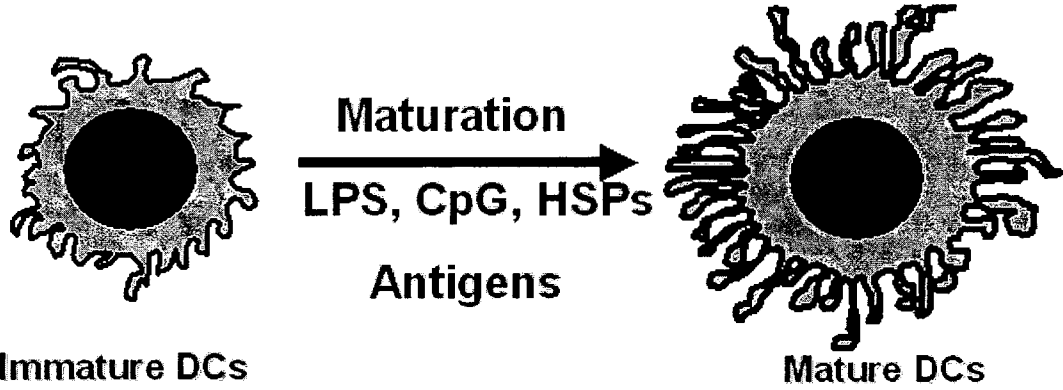
	Immature DCs		Mature DCs
+	Antigen Presentation		+++
++	Antigen Uptake Receptors		+/-
++	Endocytic Activity		+/-
+/-	Motility		++
+	Adhesion Molecule		+++
+	Costimulatory Molecules		+++
-	Secretions of (IL-12,T cell Attractant)	+++
-	CD83		+
-	Leukocyte Differentiation Antigens	-

Figure: 1.5: Status of DCs in their immature and mature state.

Status of DCs in their immature and mature (activated) states along with various properties. Activation and functional maturation can be influenced by various stimuli (LPS, Bacterial DNA, dsRNA, TNF etc). Properties as shown are generalized and not necessarily uniform for DCs derived from different sources.
Reference: (Timmerman and Levy, 1999)

The cross talk between NK cells and DCs is bi-directional because NK cells also induce DCs maturation (Piccioli et al., 2002). Activated NK cells increase the production of TNF-α and IL-12 by DCs that induces DCs maturation in a paracrine manner (Vitale et al., 2005). NK-DCs interaction results in IL-18 secretion by DCs at the synaptic cleft and the release of the DCs maturation factor HMGB1 by the NK cells (Semino et al., 2005).

High numbers of tumor-infiltrating DCs are associated with better prognosis and reduced metastases in cancer patients (Osada et al., 2004). Tumor-infiltrating NK cells produce IFN-γ, which activates DCs upon tumor-infiltration. IFN-γ activated 'killer DCs' along with activated NK cells mediated much more rigorous anti-tumor immune responses in the tumor mass (Gerosa et al., 2002; Kang et al., 2004; Sakakura et al., 2005).

DCs and NK cells also interact via multiple TNF-family molecules (Makarenkova et al., 2005). DCs mediated IFN-γ secretion by NK cell required DCs contact; DCs-TNF and NK-cell TNFR2 are required for DCs-mediated NK cell proliferation and cytotoxic activity (Xu et al., 2007). DCs protect NK cells from oxygen radical-induced inactivation or apoptosis (Thoren et al., 2007). mDCs subsets have distinct roles for the activation and viability of human NK cells *in-vivo* (Munz et al., 2005a). Fernandez et.al showed that Flt3 ligand expanded DCs activate NK cells, leading to the regression of MHCI⁻ tumor that confers a unique function for DCs to eradicate tumor *in-vivo*, and further define a crucial NK cell function 'licensed' by DCs to kill NK-sensitive tumor cell targets (Fernandez et al., 1999). NK cell dependent DCs activation has also emerged as a unique maturation stimulus for inducing an effective CD8^{+} T cell responses (Mailliard et al., 2004).

These bi-directional cross talks between NK and DCs have attained a greater interest since this rendezvous may lead to a more competent and concerted action of other immune cells against various disorders *in-vivo*.

1.3 PRESENCE OF NK CELL RECEPTORS ON MYELOID CELLS AND DCs

NK cells recognize activating ligands on tumor cell through activating receptors and only if tumor lacks or shows reduced expression of MHC I then the killing of the tumor cells are performed, since inhibitory receptor interacts with MHC I on targets and aborts the NK cell activation. However, NK cell cytotoxic function requires the balance between inhibitory and activation receptor function and operates in accordance with 'Missing self hypothesis' (Lanier, 2005a).

A number of cell surface glycoproteins have been referred to as "NK receptors" either for historical reasons (they were first identified on NK cells) or because they are predominantly expressed on NK cells (Lanier, 2007). However expression of both inhibitory and activation NK cell receptors are also shown on DCs. Inhibitory receptor Ly49Q (a lectin-type killer cell inhibitory receptor), of Ly49 family of NK cell receptors, which displays a high degree of conservation and a unique expression pattern is also found on myeloid cells, including pDCs. Ly49Q of pDCs efficiently binds H-2K$^{(b)}$ inhibitory ligand on normal cells, like NK cells. Additionally Ly49Q serves as a marker to identify precursor form of pDCs that participate in innate immunity (Tai et al., 2007). Expression of functional activating NKR-P1 receptor was found on rat killer splenic (Josien et al., 1997) and BMDCs (Brissette-Storkus et al., 2002). Cross-linking of NKR-P1 with mAb-3.2.3, activated rat DCs to induce enhanced apoptosis in tumor targets (Alli et al., 2004). Recently, NKG2D receptor has been shown on DCs from mouse and rat that generates effective antitumor immune responses (Alli et al., 2004; Chan et al., 2006). Nevertheless, apparent expression of NKG2D on myeloid cells has also been observed (Y. Zhu and Lewis Lanier, unpublished observations) (Spits and Lanier, 2007). Although expression of both activating and inhibitory receptors are found on DCs yet it requires to be established if DCs mediated direct killing of tumor cells also follows 'MHC-restriction' as in the case of NK cells.

NK cells express various c-type lectin-like receptors and their study has provided new insights into the mechanisms of immune recognition of transformed or infected host cells. The characteristic feature of c-type lectins is the presence of a c-type lectin-like domain 'CTLD' fold (Weis et al., 1998), which is usually derived from six conserved cysteine residues. Type-V c-type lectins that consist of the type-II transmembrane, single CTLD-containing non-classical molecules include all the myeloid-expressed NK-like c-type lectin-like receptors (NKCL). Group-V NKCLs are usually encoded by six exons and shares a common structure, comprising an extracellular CTLD, a stalk region of variable length, a transmembrane region and a cytoplasmic tail, which may contain signaling motifs (Kanazawa, 2007; Santis et al., 1994).

Natural killer (NK)-like CLRs has diverse ligands and cellular functions on myeloid cells in contrast to NK counterparts. These receptors play important roles in both innate and adaptive immunity through their ability to modulate myeloid cell function (Pyz et al., 2006). On APCs, some of these receptors may contribute to the activation and maturation of lymphocytes (Grunebach et al., 2002). Although for myeloid-expressed NKCLs, the ligands are unknown and their biological functions are poorly understood. The numbers of activation

and *inhibitory* NKCLs have increased considerably and many novel members are still being characterized. The list of such NKCLs is described in table 1.2 (Pyz et al., 2006).

Identification of NKG2D on DCs further adds the presence of potent tumor recognition receptor on the myeloid cells. Although NKCLs on NK cells primarily detect transformed or virally infected cells; the NKCLs appear to display divergent functions on myeloid cells and DCs. However, beyond their initial characterization, little is known about their physiological functions *in-vivo*. In addition, the inhibitory and activation mechanisms triggered by these receptors appear to be novel, but are mostly undefined (Pyz et al., 2006).

1.3.1 NKG2D/NKR-P2 Receptor

NKG2D (Natural killer group2, member D, also called KLRK1) is a type-II, c-type lectin like tumor recognition receptor, expressed as homodimer on various immune cells (Raulet, 2003). NKR-P2 (Natural killer receptor-protein 2) is the rat ortholog of human NKG2D and shows 59.9% amino acid (AA) homology with human NKG2D (Berg et al., 1998). *nkg2d* gene is located in mouse chromosome 6 (Ho et al., 1998), and in human chromosome 12 (Renedo et al., 2000). The extracellular portion of NKR-P2 contains three potential sites for N-linked glycosylation. Depending on two possible initiation codons, NKR-P2 contains 215 or 227 amino acids, with molecular mass of 24.4 or 25.8kDa. NKG2D is distantly related to other members of NKG2 family receptors (NKG2, -A-C-E) sharing a low amino acid sequence homology (Berg et al., 1998).

1.3.1.1 Versatile expression of NKR-P2/NKG2D on immune cells

Human NKG2D is constitutively expressed on NK cells, $CD8^+\alpha\beta$ T cells, $\gamma\delta$T Cells (Jamieson et al., 2002), $CD4^+$T cells (Allez et al., 2007; Groh et al., 2003; Saez-Borderias et al., 2006), and on human myeloblastic cells (Guilloton et al., 2005). Enhanced expression of NKG2D is reported on $CD4^+CD28^-\alpha\beta$ T cells in rheumatoid arthritis patients (Groh et al., 2003). In mouse, NKG2D expression is reported on all NK cells, dendritic epidermal $\gamma\delta$T Cells, some splenic $\gamma\delta$T Cells, activated $CD8^+$T cells (Jamieson et al., 2002; Nitahara et al., 2006), DCs (Chan et al., 2006), activated Mϕ (Diefenbach et al., 2003; Diefenbach et al., 2000). In rat NKG2D/NKR-P2 is expressed on NK cells, resting $CD4^+$, $CD8^+$T cells (Berg et al., 1998), DCs (Alli et al., 2004), Mϕ and MOs (Baba et al., 2006). In RT-PCR analysis, NKG2D transcripts were also found on pig NK cells, peripheral blood lymphocytes, pulmonary alveolar Mϕ, and in MOs (Yim et al., 2001). NKG2D expression on human NK

Myeloid NKCL Receptors	Distribution	Ligands	Function
LOX-1 (Lectin-like oxidized low-density lipoprotein receptor-1)	DCs, Macrophages, Endothelial cells, Platelets	Oxidized LDL, Hsp70, *S.aureus*, *E.coli*, Apoptotic cells	Oxidized LDL pro-inflammatory responses, oxidative stress
CD69	DCs,Activated lymphocytes, Neutrophils, Monocytes, Macrophages, NK and B cells, Platelets,	Unknown	Cytokines release, Microbial Stimuli, Activation of immune cells.
MDL-1 (Myeloid DAP12-associating lectin 1)	DCs, Macrophages, Monocytes,	Unknown	Maturation status of cells, Mycobacterial infection, LPS, TNF-α, IFNγ
MICL (myeloid inhibitory c-type lectin-like receptor) or DCAL-2(DCs-associated C-type lectin 2)	DCs, Myeloid cells, NK and T cells, Macrophages	Unknown	Participate in LPS mediated DCs maturation.
Dectin-1/BGR(β-glucan receptor BGR)	DCs, Macrophages, Monocytes, Neutrophils, Subsets of T cells	β-1,3 and β-1,6-glucans, zymosan, *Calbicans, S. cerevisae, P. carinii, A. fumigatis, C. posadasii* ligand on T cells	Up-regulated by PMA , IL-4 , IL-13, GM-CSF. Down-regulated by IL-10, TNF-α,IL-1β
CLEC-1 CLEC-2	DCs, Myeloid cells, Platelets	Unknown, Rhodocytin for human.	Unknown
Ly49q	DCs, NK, NKTcells, Activated macrophages	Unknown	Maturation status of cells, IFN-α or IFN-γ stimulation
KLRF-1 (Killer cell lectin-like receptor, subfamily F, member 1 (NKp80)	PBLs, NK cells, T cell subsets, Myeloid cells	Unknown ligand on T cells	Unknown

Table 1.2. List of NK-c-type lectin like receptors on myeloid cells and   DCs. Reference: (Pyz et al., 2006 ;Chen,C.H., et al., 2006)

cells decreases significantly with age (Sundstrom et al., 2007). The expression of NKG2D on various immune cells is shown in table 1.3.

Although the role of NKG2D is established well in NK cells, now it will be appropriate to say that NKG2D/NKR-P2 is preferentially expressed by mammalian lymphocytes with cytotoxic potential (Coudert and Held, 2006).

1.3.1.2 NKG2D/NKR-P2 mediated signal transduction

NKG2D is a disulfide-linked homodimer and bears only a short cytoplasmic region that does not contain any signaling motif in humans and mice (Vivier et al., 2004). In rat the cytoplasmic region of NKG2D/NKR-P2 is predicted to contain six putative protein phosphorylation sites i.e. $R_{39}RRS$ and $R_{40}RSS$ (cAMP and cGMP-dependent protein kinase), T_{29} (protein kinase C), and T_{16} SQE, S_{42} SIE and S_{43} IEE (Casein kinaseII). Four of these sites are contained in the sequence $R_{39}RRSSIEE$, present within the rat specific exon (Berg et al., 1998). In rat no evidence of alternative splicing of NKR-P2 was reported (Berg et al., 1998), whereas in human and mice NKG2D is expressed as NKG2D long and NKG2D short form (Diefenbach et al., 2002). NKG2D cytoplasmic tail associates with signal transducing adapters via amino acid of opposite charges in transmembrane region. NKG2D can associate with DNAX-activating protein of 10kDa (DAP10), which contains an YNIM signaling motif, or with DAP12 that contains ITAM motif (Garrity et al., 2005; Rabinovich et al., 2006).

Differential association of NKG2D with distinct signaling adapters leads to stimulatory or co-stimulatory signals in cells. NKG2D ligand binding leads to association of receptor with adapters DAP12 or DAP10. DAP12 recruits ZAP-70 and syk protein tyrosine kinase, whereas DAP10 recruits p85 and Grb2 (Gilfillan et al., 2002; Upshaw et al., 2006). Expression of DAP10 and DAP12 has also been shown in DCs (Chan et al., 2006; Wu et al., 1999). Recent evidences suggest that NKG2D ligation leads to multiple signal transductions leading to the activation of PI3 kinase, ERK kinase, JNK phosphorylation, STAT5, MEK1/2,Vav1, Rho GTPase and PLC-γ activation (Chen et al., 2007; Long, 2002; Upshaw and Leibson, 2006), and NF-κB activation (Regunathan et al., 2005).

1.3.1.3 NKG2D/NKR-P2 ligands and their diversity

NKG2D recognizes various distinct ligands, which display a degenerate recognition system to accommodate a broad range of interacting proteins (Strong and McFarland, 2004). NKG2D binds to carbohydrate GlcNAc, GalNAc and high mannose N-glycans when

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Immune cells	Human	Mouse/Rat
NK cells	All NK cells	All NK cells
TCR$\alpha\beta^+$ T cells	Naive, Activated and memory CD8$^+$T cells	Activated and memory CD8$^+$ T cells in mouse. Rat resting CD4$^+$, CD8$^+$ T cells express NKG2D transcript.
TCR$\gamma\delta^+$ T cells	Most of the blood and Intestinal intraepithelial lymphocytes, TCR$\gamma\delta^+$ T cells	25% of TCR$\gamma\delta^+$ T cells in spleen
NKT cells	Not determined	Large fraction of NKT cells in mouse, Not determines in rats
Dendritic cells	Monocyte derived DCs	Mouse and Rat BMDCs, Mouse and Rat splenic DCs, Rat lymph nodes DCs
Macrophages	Not determined	Transcript present in CD8$^+$ macrophages, Activated macrophages.
Monocytes	Not determined	Not determined in mouse, Transcript present in rat CD8$^+$ monocytes.

Table 1.3. List of NKG2D expressing immune cells of human and murine origin.
Reference: (Alli et al., 2004; Baba et al., 2006; Coudert and Held, 2006; Korthals et al., 2007).

presented in the form of neo-glycoproteins (Bezouska, 2004), however the functional role of these carbohydrate ligands has not been elucidated.

NKG2D shows promiscuous interactions with a diverse array of ligands. NKG2D recognizes a variety of highly diverse MHC I-like molecules. NKG2D interacts with multiple ligands that are usually upregulated on stressed cells and mounts antitumor immune response (Gonzalez et al., 2006). A range of NKG2D ligands have been identified which include MICA/MICB and ULBPs proteins in humans (Bahram et al., 2005). Like MHC I, MICA/B are transmembrane proteins with three immunoglobulin (Ig)-like domains. Overall homology of MICs to MHC I is only 25% and MICs do not associate with β2-microglobulin or peptides in contrast to MHC I (Bahram et al., 1994). Rae1, H60 and MULT1 are the other MHC I-like ligands for mouse NKG2D (Carayannopoulos et al., 2002a; Cerwenka and Lanier, 2003).

Thus NKG2D ligands exhibit an enigmatic diversity for the recognition components of the receptor. NKG2D recognizes these diverse ligands, which have little sequence homology (25-40% sequence homology among families), and distinct different domain organizations. Many NKG2D ligands posses homologous MHC I- like α1 and α2 domains, MIC contains additional α3 and transmembrane domain, whereas ULBPs, rae1 and H60 families are GPI anchored proteins (Raulet, 2003). Human cytomegalovirus-encoded UL16 discriminates MICs by their α2 domains (Spreu et al., 2006). Recently, a viral encoded protein OMCP (orthopoxvirus MHC I-like protein), which lacks transmembrane anchorage properties, was shown as a secretory ligand of NKG2D that does not show sequence homology with previously known ligands of NKG2D. However OMCP acquires the MHC-I-like conformation, which was established through bioinformatic tools. This virally encoded OMCP acts as a paracrine inhibitor of NKG2D upon release and aborts the natural interactions with other ligands, thus participates in immune evasion (Campbell et al., 2007). Thus NKG2D ligands are variable in both their amino-acid sequence and domain structure.

NKG2D interacts with distinct ligands with varied affinities, ranging from 4 to 800 nM (Carayannopoulos et al., 2002b). NKG2D and NKG2D-ligand co-crystal structures revealed that NKG2D contains a large, flat surface for the binding of distinct ligands. NKG2D has a symmetric binding surface but it binds to the asymmetric surface of ligands, exhibiting the tolerance against various ligands upon complex formation (Li et al., 2001). Upon ligand binding NKG2D flat surface undergo conformational adjustment, by which identical NKG2D residues adopt different conformations in the receptor subunits. This structural plasticity enables the binding of distinct ligands by the common elements of the NKG2D platform (McFarland et al., 2003).

It is now accepted that NKG2D interacts with dissimilar ligands by induced fit mechanism. Biophysical studies have explored that H-bonds, hydrophobic interactions and salt bridges are the crucial components, which contribute to maintain the overall shape complementarity for induced fit recognition of NKG2D ligands. Accordingly various energetic aspects of multispecific immune recognition by NKG2D has been discovered by crystallographic structure (O'Callaghan and Jones, 2003), which show that NKG2D uses dimeric surface for ligand binding, which is a characteristic of c-type lectin like domain (Strong and McFarland, 2004). Moreover this conformational plasticity was also confirmed by the co-crystal structure of NKG2D and its MHC I-like ligand ULBP3 (Radaev et al., 2001).

Although MHC I-like proteins were characterized as functional ligands of NKG2D, various other proteins were expected to be the ligands of NKG2D that include signal-peptide derived fragments, oxidized lipids and heat shock proteins (Bezouska, 2004). Recently HSP70 family members HSPA1A, heat shock 70kDa protein8, Grp78 and other HSP70 family members have been shown to be the ligands of NKG2D (Theriault et al., 2006).

1.3.1.4 Regulation of NKG2D ligand expression

NKG2D ligand expression marks the stressed or infected cells or tumor cells (Mistry and O'Callaghan C, 2007). First study to investigate the regulation of NKG2D ligand revealed that *MICA/B* promoter resembles the hsp70 promoter and stress inducible nature of NKG2D ligands (Groh et al., 1996; Venkataraman et al., 2007). NKG2D ligands have diverse expression patterns on tumor cells (Pende et al., 2002). MICA surface expression was reported during cytomegalovirus infection (Groh et al., 2001). Many tumor cell lines express MICA at surface that suggests its association with transformation (Bauer et al., 1999). MICA/B expression was detectable in gastrointestinal epithelial cells (Groh et al., 1996), synovial cells of rheumatoid arthritis patients (Groh et al., 2003). Another human NKG2D ligand ULBP is expressed on various tumor cell lines of epithelial and hematopoietic origin (Salih et al., 2003).

Like human NKG2D ligands Rae1 and H60 expression are reported in various tumor cell lines (Cerwenka et al., 2000). Retinoic acid (differentiation agent) induces the expression of Rae1 in embryonal carcinoma, consistent with the idea that NKG2D ligands are developmentally regulated (Nomura et al., 1996) and induce expression of MICA/B in hepatoma (Jinushi et al., 2003). Influenza or Sendai virus infection leads to induction of NKG2D ligand expression in human Mφ (Siren et al., 2004), TLR agonist also induces

expression of NKG2D ligand in murine macrophage (Hamerman et al., 2004). Recently, expression of UL16 binding protein-1 (ULBP-1) on mDCs was also found in T cell areas of LNs and also reported to appear upon maturation (Schrama et al., 2006). Expression of several oncogenes (K-ras, Akt, c-myc, E6, E7, E1A or Ras V12) or the lack of a tumor suppressor (p53) does not induce NKG2D ligand expression in primary ovarian cells *in-vitro*. This suggests that transformation of primary cell is not an essential prerequisite for NKG2D ligand induction (Gasser et al., 2005).

Differentiation promoting drugs (5-aza-2'-deoxycytidine, trichostatin A, vitamin D3, bryostatin-1, and all-trans-retinoic acid) up-regulate NKG2D ligand expression and enhance the susceptibility of acute myeloid leukemic cells to NK cell-mediated lysis (Rohner et al., 2007).

The DNA damage response causes cell-cycle arrest and induction of DNA repair functions for the survival of modestly damaged cells. However, severely damaged cells are induced to undergo apoptosis. DNA damage response is activated very early during tumorigenesis, providing evidence that the DNA damage response could function as a barrier in early tumorigenesis (Gasser and Raulet, 2006a). Recently, DNA damaging agents are shown to induce NKG2D ligands in fibroblasts and in lymphoid cells. DNA damage induces phosphorylation of ATM/ATR kinases. These kinases induce phosphorylation of additional kinase leading to cell-cycle arrest and apoptosis. Additionally, these pathways also induce upregulation of NKG2D ligands (Gasser et al., 2005; Gasser and Raulet, 2006b).

Certain cytokines, especially the IFNs regulate expression of specific NKG2D ligands on murine tumors (Bui et al., 2006). It has also been shown that non-lethal heat shock induces NKG2D ligand expression in various cancer cell lines along with the upregulation of heat shock proteins (Kim et al., 2006). These evidences insight that the sophisticated regulation of NKG2D ligand expression is important since inappropriate expression in normal tissues may favour autoimmune disorders, whereas failure to upregulate the NKG2D ligands in pathological conditions or on tumor cells may lead to cancer development.

Overall NKG2D ligand expression on tumor cells or on infected cells is regulated by multiple factors under distinct conditions. It is conceivable that the expression of different ligands is governed by distinct cellular or environmental stress that can direct effective anti-tumor immune response. The diversity in the regulation of NKG2D ligands also offers to break the common strategies adopted by the stressed cells to evade the NKG2D detection (Mistry and O'Callaghan C, 2007).

1.3.1.5 Role of NKG2D in tumor immunosurvillence

Emerging data suggest that NKG2D holds a strong role in immunosurvillence. NKG2D ligands are rarely expressed on healthy tissues whereas tumor cells frequently expresses NKG2D ligands on the cell surface (Pende et al., 2002; Romanski et al., 2005). Animals reject NKG2D ligand transfected tumors vigorously (Busche et al., 2006; Diefenbach et al., 2001). NKG2D plays a significant role in immunosurveillance against chemically induced tumors (Girardi et al., 2001). Chemically induced skin tumors frequently express NKG2D ligand, whereas they remain absent from normal skin cells (Smyth et al., 2004). NKG2D ligand expressing tumors contain larger lymphocytic infiltrates as compared to NKG2D ligand-negative tumors (Vetter et al., 2002). A higher level of NKG2D ligand expression also correlates with a better prognosis in colorectal cancer (Watson et al., 2006). NKG2D function protects the host from tumor initiation (Smyth et al., 2005). NKG2D mediated recognition and perforin effector function mediate effective cytokine immunotherapy of cancer (Smyth et al., 2004). The ability of NKG2D to identify and destroy tumor cell, acts as primary defense mechanism against cancer. Various evidences suggest that NKG2D participates adequately in immunosurvillence, however dysregulation of NKG2D function has also been demonstrated in cancer patients (Groh et al., 2006; Wiemann et al., 2005; Kaiser et al., 2007;Groh et al., 2005).

1.4 AGONISTIC mAbs AND THEIR THERAPEUTIC USES

The typical antibody consists of Ag binding fragments (Fabs), which are linked via a hinge to a constant (Fc) region. On immune cells mAb binds to specific epitope (receptor) and either blocks or evokes a functional response depending on their binding affinity or avidity. Thus mAb acts as artificial ligand for the receptors and is now defined as a new family of drugs that augment immune responses (Alfaro et al., 2006).

Upon cross-linking of receptor with agonistic mAb, the signaling cascade associated with the receptor can be determined; hence agonistic mAb serves as a ligand to decipher the function of the receptor in the absence of a functional ligand (Melero et al., 2007). It is highly advantageous that mAbs of defined specificity can be produced in high amounts (Kohler and Milstein, 1975) and has the potential for the management of various diseases, including malignancy. The key property of mAbs to be used, as therapeutic tools is their high avidity ligands to the receptors (Gray et al., 2006).

In general the responses of specific mAbs or respective ligands remains identical. However few reports argue that mAbs mimic the natural ligand of the receptors, e.g. Fas

receptor triggers apoptosis when stimulated by Fas-ligand or anti-Fas agonistic Ab. Activated Fas-receptor oligomerizes into microaggregates, and the microaggregate formation has been reported by agonistic anti-Fas mAbs, but not with Fas ligand. These results suggest that subtle differences exist in the functional response triggered by anti-Fas agonistic Abs and FasL (Legembre et al., 2003). Similarly, bivalent Ab could block ligand-receptor interactions, whereas petavalent IgM efficiently cross-link cell surface targets, and evoke physiological responses.e.g. IgM Ab against B7-DC receptor on DCs induces a gene expression program (Blocki et al., 2006). Jamieson et.al showed that the cross-linking of NKG2D with immobilized anti-NKG2D mAb MI-6 triggers murine NK cells and primed Mϕ (Jamieson et al., 2002), whereas similar effects could not be obtained for human NK cells by immobilized anti-NKG2D mAb (Wu et al., 2000). It was therefore attributed that NKG2D signals differently in human versus mouse NK cells, or the distinct results reflect experimental differences, such as the use of an NK cell line in the human studies or the use of different concentrations of anti-NKG2D Ab (Jamieson et al., 2002). NKG2D cross-linking is not directly stimulatory for primed mouse $CD8^+$ T cells, which express abundant cell surface NKG2D, whereas cross-linking of NKG2D enhances the proliferation of $CD8^+$ T cells induced by TCR triggering, in line with evidence that human NKG2D provides primarily a co-stimulatory signal for T cells. These results attributed that NKG2D signals differently in distinct immune cell types (Ehrlich et al., 2005; Jamieson et al., 2002).

The principle of immunotherapeutic mAbs is to block or activate similar type of receptors expressed on a cell type *in-vivo*. However *in-vivo* function may differ if one such receptor is expressed on multiple cells types, due to the distinct signaling machinery. CD40 is a 48 kDa transmembrane glycoprotein belonging to the TNFR superfamily. It is also expressed on the surface of B-cells, and DCs (Quezada et al., 2004). CD40 signaling on DCs brings about 'licensing' of the DCs with enhanced Ag presentation, cytokine release and survival. Function of agonistic mAb is dependent on the maturation stage of immune cells. For example, anti-CD40 mAb induces maturation of iDCs that drives adaptive immune responses whereas anti-CD40 mAb induces apoptosis in mDCs, which reflects surprising dual role of agonistic mAbs on DCs (de Goer de Herve et al., 2005). The differential affects of mAbs are an intriguing issue, e.g. Agonistic anti-CD40 mAb induces the activation/proliferation of primary B cells whereas it exhibits apoptotic effects on some CD40-expressing tumor cells. For the differential behavior of anti-CD40 mAbs, various reasons are acknowledged, that include auto-induction by TNF, inappropriate association or

the inability of distinct cells to attenuate receptor signaling by agonistic anti-CD40 mAb (Francisco et al., 2000). Although the differential binding properties of mAbs are confirmed, yet no reasons are provided leading to puzzling situation. e.g. Anti-CD40 mAbs, SGN-14 enhances the binding of CD40 to CD40 ligand on T cells, whereas other anti-CD40 mAbs, G28-5a and M3 inhibit the binding of CD40 ligand to CD40. It is speculated that SGN-14 induces a conformational change that stabilizes CD40 multimerization in the presence of CD40 ligand. The contrasting effects of the agonistic CD40 mAb SGN-14 was also demonstrated that increases the binding of CD40L to CD40 in soluble conditions whereas SGN-14 inhibits the binding of soluble CD40 to membrane bound CD40 ligand (Francisco et al., 2000).

The agonistic and antagonistic properties of CD40 mAb G28-5 are shown to be dependent on binding valency (Ledbetter et al., 1994). However SGN-40, a humanized anti-CD40 Ab, induces cytotoxicity in human multiple myeloma cells. SGN-40-mediated cytotoxicity is associated with up-regulation of cytotoxic ligands of the TNF family (Fas/FasL, TNF-related apoptosis-inducing ligand, and TNF-α (Tai et al., 2004). In animal tumor models, antibodies against a number of targets, including CD40 (French et al., 1999), 4-1BB (Melero et al., 1997), OX40 (Weinberg et al., 2000), and CD25 (Onizuka et al., 1999), have been shown to provoke powerful tumor specific responses capable of eradicating established tumor.

In general, pharmacokinetics nature of mAbs are considered very favourable because they are protease resistant, stable in plasma, and generally endowed with longer half-life. The aim in cancer immunotherapy is to employ the specificity of the immune system to provide an effective, less toxic, treatment compared to the conventional therapies. A new approach, which employs the use of agonistic mAbs to boost weak endogenous anti-tumor immune responses are confirmed to be therapeutic. Clinical experience of this group of mAbs is limited and there are obstacles to translate the promising effects into therapies for patients. However, several immunostimulatory mAbs have now entered clinical trials and showing promising results. The immunotherapeutic results in patients are highly encouraging, and also being included in combination with other immunotherapies and chemotherapies (Gray et al., 2006).

1.5 HEAT SHOCK PROTEINS AND IMMUNOLOGICAL FUNCTIONS

Intracellularly HSPs (heat shock proteins) constitute upto 5% of total cellular proteins and their levels increases upto 15% or more under cellular stress (Srivastava, 2002b). HSPs are localized in various cellular compartments and their function related to protein folding, transport and repartition; and their protective functions increasing cell resistance to stressful conditions have been extensively studied and established (Hartl, 1996; Schlesinger, 1990). HSPs of mammalian cells can be classified into several families of sequence related proteins. The principal mammalian HSPs, based on protein expression levels, are cytoplasmic/nuclear proteins with molecular masses of 25 kDa (hsp25), 70 kDa (hsp70), 90 kDa (hsp90), and 110 kDa (hsp 110); intracellularly, these HSPs have important physiological function in folding and transport of newly synthesized proteins and prevent degradation of nascent proteins (Srivastava, 2002a).

Members of various HSPs families are highly immunogenic (Multhoff and Hightower, 1996). In order to distinguish different function of HSPs in eliciting immune responses four paradigms have been proposed (Srivastava, 1994).

1. HSP as classical species-specific foreign Ags: Despite high sequence homology, all HSPs contain a region that acts as foreign epitopes for the host's immune system.

2. HSP as self-Ags that are expressed in tissue specific manner.

3. HSP as example of molecular mimicry between HSP and self-Ags.

4. HSP as presenting molecule for foreign Ags in a non-covalent complex.

In all the above-proposed paradigms, HSPs elicit an immune response because they are located on the cell surface (Multhoff and Hightower, 1996).

1. 5.1 Cell Surface expression of HSPs in stressed and tumor cells

The immunogenic function of endogenous HSPs was recognized way back that demonstrated the role of hsp-asociated peptides as tumor Ags, which elicit anti-tumor immunity (Srivastava and Das, 1984; Srivastava and Old, 1989; Srivastava et al., 1994). Cell surface localization of various HSP families is recognized on stressed cells recently (Altmeyer et al., 1996; Di Cesare et al., 1992; Jang and Hanash, 2003; Multhoff and Hightower, 1996; Roigas et al., 1998). In most cases, an organism cannot recognize tumor cells as foreign. Inflammatory sites are rarely found in a tumor area before the beginning of massive necrosis, even in large tumor. However, it became obvious that the immune system

may start recognizing cancer cells expressing elevated amounts of HSPs (Guzhova and Margulis, 2006). Moreover, many HSPs were reported on the cell surface. Few examples of the surface HSPs expression are summarized below.

HSP25: Hsp25 surface expression is shown on mammary adenocarcinoma that grows faster in contrast to low hsp25 expressing cells *in-vivo*. Hsp25^{high} 4T1 carcinoma metastasized to lungs more aggressively. Interaction between host effector cells and tumors expressing high level of surface hsp25 results in enhanced immune responses (Bausero et al., 2004). Silencing of *hsp25* gene aborts the migration capability of the highly metastatic murine 4T1 breast adenocarcinoma cells (Bausero et al., 2006).

HSP60: Hsp60 is primarily a mitochondrial protein, but its surface expression has been found on Burkitt's lymphoma (Cicconi et al., 2004; Kaur et al., 1993). Hsp60 surface expression is also found on the endothelial cells upon heat shock and upon treatment with TNF-α, hsp60 surface expression makes these cells susceptible to complement-dependent lysis (Xu et al., 1994). Surface hsp60 also participates in the adhesion of metastatic breast cancer cells (Barazi et al., 2002). Leukemic CD4-positive T-cell line also expresses hsp60 on cell surface (Soltys and Gupta, 1997). Surface expression of hsp60 is found on oral cancer cells (Laad et al., 1999), breast, lung carcinoma cells (Barazi et al., 2002), spleen and liver cells derived from mice infected with *Listeria monocytogenes* (Belles et al., 1999) and also on the lymphocytes of rheumatoid arthritis patients (Sato et al., 1996). In Daudi cells, it was demonstrated that both N-terminus and c-terminus of the hsp60 are exposed outside the cells (Cicconi et al., 2004; Kaur et al., 1993). Hsp60 surface expression also ocurs upon LPS treatment to neutrophils, suggesting the infection induced surface expression (Hirsh et al., 2006). In unstressed HUVEC (human umbilical venous endothelial cells) hsp60 is primarily located in mitochondria whereas in stressed cells, hsp60 expression was detected on cell surface. Three highly sensitive methods (Confocal laser microscopy, atomic force microscopy and flow cytometry) have confirmed hsp60 surface localization on stressed HUVECs. A non-unifrom, patchy distribution of hsp60 in clusters was found on HUVEC surface, which might be associated with rafts. It is also presumed that hsp60 on stressed cells acts as a danger signal *in-vivo*, triggering inflammatory disease such as arteriosclerosis (Pfister et al., 2005).

HSP70: Intracellularly hsp70 binds and stabilizes non-native conformation of other proteins or enables translocation across the membrane (Multhoff et al., 1998). In the mid 1990s,

several reports indicated that hsp70 could be found on the surface of malignant cells, which was not observed in normal cells (Botzler et al., 1998b; Multhoff et al., 1995b). The intracellular protective function of hsp70 is related to its ability to promote folding of nascent polypeptides. Many types of cancer cells contain high amounts of hsp70, whose protective capacity may pose a problem for therapy in oncology. However surface hsp70 expression was reported on various tumors. Therefore, surface hsp70 is an important structure that could be recognized by immune cells (Guzhova and Margulis, 2006). Intracellularly, hsp70 protects tumor cells from stress factors, which makes anti-cancer therapy ineffective. However, expression of hsp70 on the surface of cancer cells may induce anti-tumor immune responses (Guzhova and Margulis, 2006). Based on the proposed mechanisms for surface hsp70 there were attempts to create an anti-cancer vaccine. Figure 1.6 describes the role of surface HSP in immune recognition. Several studies demonstrated that tumors of head and neck cancer patients (Kleinjung et al., 2003), colorectal cancer, lung carcinoma, neuronal tumor, pancreatic carcinoma, and leukemic blast cells, (Farkas et al., 2003; Ferrarini et al., 1992; Multhoff et al., 1998; Pfister et al., 2007) show surface expression of hsp70. Hsp70 plasma membrane expression is also reported on the primary tumor biopsy material and on the BM of leukemic patients (Hantschel et al., 2000). Differential hsp70 plasmamembrane expression is reported on primary human tumors of the patients with severe immunodeficiency (Botzler et al., 1998b). Surface expression of hsp70 increases in some tumor cells by passive hyperthermia (Multhoff, 1997; Roigas et al., 1998).

It has been shown that hsp70 surface expression is restricted to tumor cells but not on normal cells, indicating that hsp70 might act as a tumor specific target structure for effector immune cells (Multhoff et al., 1995a). Accordingly it was demonstrated that hsp70 surface expression on carcinoma cells is associated with an increased sensitivity to lysis by NK cells (Botzler et al., 1996; Gross et al., 2003c), which confirms hsp recognition by immune cells. Hsp70 on tumor cell surface is also confirmed as a recognition structure for NK cells (Multhoff et al., 1997). Hsp70 colocalizes with Bag-4 (a silencer of death domain) on the plasmamembrane of tumor cells. Surface expression of hsp70 elevates in Bag-4 over-expressing HeLa cells in comparison to untransfected cells (Gehrmann et al., 2005). In EL-4 lymphoma cells relative level of hsp70 cell surface expression is associated with apoptosis (Sapozhnikov et al., 2002). Hsp72 surface expression is also reported on LPS infected polymorphonuclear neutrophils but not on normal neutrophils (Hirsh et al., 2006). Inhibitors of transcription, protein synthesis and intracellular protein transport blocked the hsp72 surface expression, indicating the *de-novo* protein translocation of hsp72 in LPS-stimulated

Tumor cell

Hsp70 accumulation
in cytoplasm

Hsp70 location
onto cell surface

Resistance to
anticancer drugs

Immune response
induction
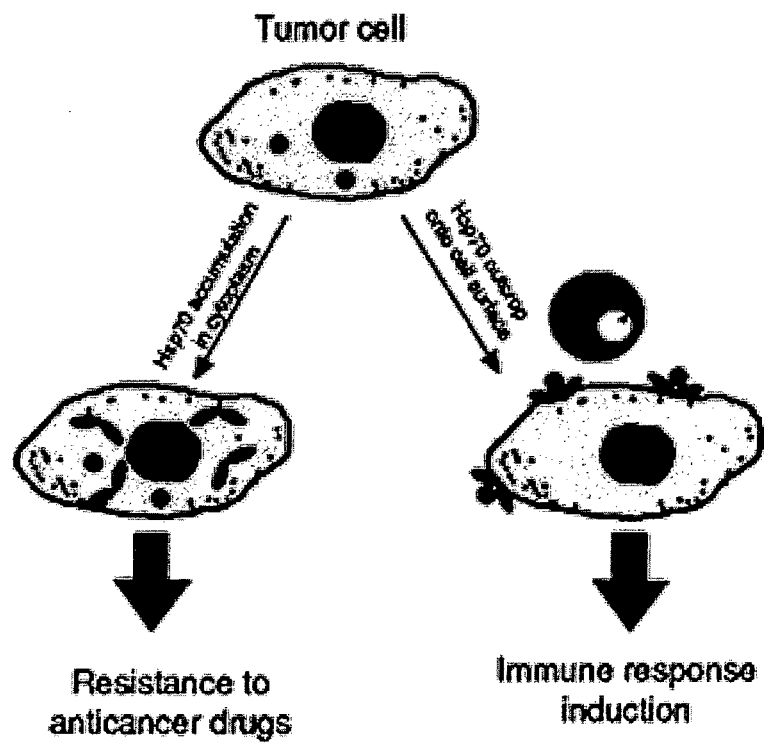
Figure.1.6: Surface hsp70 on tumor cells and its immunological function.

Many tumor cells express high amounts of Hsp70 intracellularly and therefore acquire resistance to antitumor therapy. However, in tumor cells Hsp70 expression also appears on the plasmamembrane. Immune cells can recognize the expression of Hsp70 on tumor cell surface.
Reference: (Guzhova and Margulis, 2006).

neutrophils. γδT cells directly recognize surface hsp72 on LPS-treated neutrophils and show cytotoxicity, suggesting the hsp72 recognition on cell surface (Hirsh et al., 2006). Tumor cell membrane-bound hsp70 elicits anti-tumor immunity (Chen et al., 2002). Under *in-vivo* conditions, transplantation of tumor cells subjected to non-lethal heat shock fail to form tumors in syngenic mice, whereas they form tumors in athymic nude mice, in this study inducible hsp70 is also established as a marker of tumor immunogenicity (Clark and Menoret, 2001). In mastocytoma cells (P815) that express surface hsp70, enhance immunogenicity of tumor, as hsp70 surface expressing P815 are vigorously rejected upon transplantation *in-vivo*, in contrast to wild type P815 tumor cells. Lymphocytes from hsp70-vaccinated mice kill the wild types P815 tumor cells, and show the involvement of NK cells and CTLs in anti-tumor immune responses *in-vivo* (Multhoff et al., 1995a). Surface hsp70 on tumor cells mediates perforin-independent apoptosis by specific binding and uptake of granzyme B (Gross et al., 2003b). In acute myeloid leukemia, surface hsp70 acts as a tumor specific recognition structure for the cytolytic activity of NK cells (Gehrmann et al., 2003). Exosomes are internal multivesicular bodies that are secreted upon fusion with plasmamembrane; these are detergent soluble vesicles, with the biophysical characteristic of plasmamembrane produced by inward budding of endosomal membrane in a process sequestering proteins. The composition of surface bound proteins on exosomes reflects that of plasmamembrane of tumor cells from which they have originated. Recently, the active release of tumor exosomes is reported that contains surface hsp70 and stimulates NK cell activity (Gastpar et al., 2005). Hsp70 surface-positive tumor exosomes also stimulate migratory and cytolytic activity of NK Cells (Gastpar et al., 2005).

The mechanism by which HSPs anchor at cell membrane and shows its immune cell-activating effect remains unclear despite a great number of studies on this topic (Guzhova and Margulis, 2006). Based on staining by mAb against of C-terminal and N-terminal part of full-length hsp70, it was found that C-terminal part of hsp70 is exposed to extracellular side and at least one α-helix is important for NK cell recognition and defined as 'extracellular localized epitope' of hsp70 for NK cell response. Additionally a 14-mer-hsp70 peptide termed TKD (TKDNNLLGRFELSG) exhibits stimulatory properties for NK cells (Botzler et al., 1998a; Gastpar et al., 2004). Surface hsp70 was also found on the synovial cells derived from synovial tissue of the patients of rheumatoid and juvenile arthritis (Nguyen et al., 2006). Leukemic blasts frequently express hsp70 on cell surface and thereby present activating signals to CD94^{+} NK cells (Gross et al., 2007).

Upon treatment with anti-inflammatory drugs cytosolic hsp70 level confers resistance to further stress, whereas surface hsp70 rendered tumor cells more sensitive to the immunological attack mediated by NK cells, these results offer a rationale for combining anti-inflammatory drugs with immunotherapy in cancer therapy, which is mediated by surface hsp70 (Gehrmann et al., 2004). Hsp70 markedly increases on the surface of human Epstein-Barr virus (EBV) transformed B cells that act as a target for T cell mediated cytotoxicity and also acts as the molecule to trigger $\gamma\delta T$ cells in the early stage of tumorigenesis (Zhang et al., 2005). In contrast to solid tumors, leukemic blasts frequently express both hsp70 and HLA-E on their cell surface, which may give activating and inhibitory signals to NK cells. Gross et.al. have recently shown that neither autologous/allogeneic leukemic blasts (Hsp70$^+$/HLA-E$^+$double-positive) nor the erythroid leukemic cell line K562 (Hsp70$^+$/HLA-E$^-$) are susceptible to lysis by resting NK cells. However, IL-15/TKD stimulated NK cells initiate a cytolytic response against K562 cells and autologous/allogeneic Hsp70$^+$/HLA-E$^+$ double positive leukemic blasts. These findings indicate that the stimulatory effects of membrane Hsp70 override the inhibitory influence of membrane HLA-E expression. This also insight that cytokine/TKD-activated NK cells might be an effective strategy for eradicating Hsp70$^+$/HLA-E$^+$ double positive leukemic blasts (Gross et al., 2007).

HSP90: In HSP90 family, gp96, which resides in the endoplasmic reticulum, is expressed on the surface of certain tumor cells (Ferrarini et al., 1992). Gp96, that contains the endoplasmic reticulum retention motif (KDEL), is expressed on the surface of three different lymphoid tumor cell lines, each derived from a different spontaneously arising thymic tumor (Robert et al., 1999). Surface targeting of hsp90 family members on tumor cells induces DCs maturation and confers anti-tumor immunity *in-vivo,* which endows a principle of bridging innate and adaptive immunity for cancer immunotherapy (Robert et al., 1999). Genetically targeting gp96 onto cell surface in a transgenic mouse model, 96tm-Tg, has shown that surface expression of gp96 leads to DCs activation and spontaneous lupus-like autoimmune disease (Liu et al., 2003). Using a bone-marrow chimera approach, it has also been shown that both DCs activation and autoimmunity elicited by the cell surface gp96 are dependent on the downstream adapter protein MyD88 for signaling by Toll/IL-1 receptor (Liu et al., 2003). In B cells, surface hsp90 appears upon infection with Epstein-Barr-Virus that plays an important role in the stimulation of $\gamma\delta T$ cells (Kotsiopriftis et al., 2005). Both malignant and nonmalignant human breast cell lines express hsp70 on their surface; in contrast only

malignant cell lines express gp96 on surface. Surface gp96 also correlates with NK-mediated cytotoxicity, which was confirmed by blocking surface gp96 by specific mAb, thus surface expression of gp96 on malignant cells also has therapeutic implications (Melendez et al., 2006).

HSP110: The high molecular weight stress proteins hsp110 are highly related in sequence and structure to the hsp70 family and together is referred as "HSP70 superfamily" While individual stress proteins has been studied for several years. HSP110, has received little attention. Hsp110 has been found by sequence analysis to represent as a relative of the hsp70 family (Easton et al., 2000; Facciponte et al., 2007). Hsp110 overexpression increases the immunogenicity of murine CT26 colon tumor and suggests that increased level of hsp110 provides an immunostimulatory signal *in-vivo* (Wang et al., 2002a). Hsp105 overexpression is reported in various human tumors including, colorectal, pancreatic, adenocarcinoma, thyroid, esophageal, breast, bladder carcinoma, islet cell tumor, gastric malignant lymphoma, pheochromocytoma, and seminoma, whereas, its overexpression was only found in the testis of normal adult human tissues. Thus, hsp105/hsp110 acts as useful marker of a variety of human tumors and hsp105 may prove to be a target molecule for designing anti-tumor immunotherapy (Kai et al., 2003). Studies have also shown that vaccination with irradiated hsp110 over-expressing tumor cells can elicit a strong tumor specific CTL response and generate high number of IFN-γ secreting cells. Combined administration of Hsp110 overexpressing cells and GM-CSF producing cells, targets the DCs more efficiently and evokes better functional response (Wang et al., 2002a). Hsp110 acts as an anti-cancer vaccine when complexed to tumor Ags, and receptor mediated binding of hsp110 to Mφ and DCs is recently shown (Facciponte et al., 2007).

1.5.2 Interaction of HSPs with DCs

As the immunological function of HSPs was revealed, the intense interest in the extracellular function of HSPs is rejuvenated. HSPs are exposed to external environment to mount the immune responses in two modes. Firstly when HSPs are released upon cell death and secondly, when HSPs are expressed at the outer surface of the plasmamembrane of stressed cells and tumors (Multhoff and Botzler, 1998). HSPs are the potent activators of immune cells and induce inflammatory responses. HSPs participate in cytokine release and signal transduction (Srivastava, 2002a), and enhance Ag presentation to T lymphocytes. Surface HSPs are important in targeting cytotoxic cells (Moseley, 1998).

HSPs interaction with APCs has two consequences. Firstly, the HSP.peptide complexes are taken up by the APCs and the peptides are re-presented on MHCs of APC (Doody et al., 2004; Suto and Srivastava, 1995).Secondly, HSPs stimulate APCs to secrete cytokines like TNF-α, IL-1β, IL-12, IL-6 and GM-CSF (Asea et al., 2000; Basu et al., 2000; Chen et al., 1999; Moroi et al., 2000). HSPs also induce the release of chemokines MCP-1, MIP-1 and RANTES (Lehner et al., 2000), and NO (nitric oxide) by Mϕ and DCs (Chen et al., 1999; Panjwani et al., 2002). HSPs also upregulate maturation markers like MHC II, CD86, CD83 and CD40 on DCs (Basu et al., 2000) and induce DCs to migrate towards draining LNs (Binder et al., 2000a). Microbial hsp70 also stimulates the maturation of human DCs (Kang et al., 2004). Thus HSPs perform 'chaperoned pro-peptide' and act as 'danger signal' to DCs.

It has been shown that the peptide representation and signaling consequences require two different categories of receptors on APCs. Representation of hsp asscociated peptide requires the 'internalization receptors', whereas the direct DCs activation and maturation are induced by 'signaling receptors' (Binder et al., 2004). The multiplicity of HSPs receptor indicate specialization for individual functions: receptors such as the TLR, CD40 and CCR5 are adapted for transmembrane signaling while CD91 and scavenger receptors may play more important roles in the internalization of hsp (Calderwood et al., 2007).

Since various HSPs share homologous sequences, hence saturable surface binding, specificity, and competition with other ligands are used to define the physical asscociation of HSPs to the receptors on immune cells (Binder et al., 2000b). In addition, blocking of functions like cytokine release and representation of peptides are used to confirm the functional interaction of particular HSPs with their receptors (Basu et al., 2001).

1.5.3 Receptors of HSPs

1.5.3.1 LRP/α_2-macroglobulin receptor (CD91)

CD91 was the first identified receptor for HSPs on DCs that facilitates the process of cross-presentation. CD91 binds to gp96, hsp90, hsp70, and calreticulin on APCs (Basu et al., 2001). Already known ligand of CD91 α_2-macroglobulin, inhibits the presentation of hsp-bound peptide, which confirmed the structural and functional basis for CD91 as a HSP receptor. CD91$^{-/-}$ cell lines failed to present hsp-chaperoned peptides (Binder and Srivastava,

2004). Gp96 also binds to CD91 on some T cell (Banerjee et al., 2002). Hsp70, gp96, and calreticulin do not share structural similarity but all the three bind to CD91, which reflects the surprising ligand binding nature of CD91. Multiple ligand binding nature of CD91 may be attributed to the large multidomain 600-kDa of CD91, which also binds to additional 32 ligands, many of which have no sturctural similarity (Herz and Strickland, 2001). Mice immunized with tumor-derived gp-96.peptide reject the subsequent challenge of tumor (Binder and Srivastava, 2004) suggesting the potent role of CD91 in cross presentation. The chaperoning function of gp-96 has been shown with both full length and N-terminal sub-domain that induces DCs maturation (Biswas et al., 2006) suggesting that CD91 can bind to N-termial as well as other parts of HSPs. The structural criteria for binding of HSPs by CD91 is under investigation in various laboratories that may reveal the specific binding domain for HSPs on CD91.

1.5.3.2 CD40

CD40 is a TNF-receptor family member, present on MOs, DCs, B cells, endothelial cells, and epithelial cells. CD40 binds and intearnalizes human hsp70.peptide complex. Binding of hsp70.peptide complex to CD40 is mediated by the N-terminal nucleotide-binding domain of hsp70 in their ADP state. Binding of 'hsp70-ADP' to CD40 increases in the presence of hsp70 associated peptides (Becker et al., 2002; Millar et al., 2003; van Kooten and Banchereau, 2000). The 70 kDa mycobacterial heat shock protein (Mtb-hsp70) stimulates mononuclear cells to release CC-chemokines. Chemokine release by Mtb-hsp70, but not human hsp70, is dependent on the cell surface expression of CD40. Specific binding of CD40-transfected HEK 293 cells to Mtb-hsp70 was demonstrated by surface plasmon resonance which confirmed the physical association between these molecules (Lazarevic et al., 2003; Wang et al., 2001). Immunization with hsp70 and LCMV- derived antigenic peptides breaks the tolerance to the antigenic peptide expressed as self Ags in transgenic mice, CD40$^{-/-}$ mice fail to break this tolerance (Millar et al., 2003), which confirms the functional role of CD40 as a HSP receptor.

1.5.3.3 LOX-1, SREC-1 and FEEL-1

Scavanger receptors have been defined as receptors for modified form of lipoprotein. Various scavanger receptors are expressed on Mϕ and DCs (Murphy et al., 2005). It has been shown that scavanger receptors LOX-1, SREC-1 and FEEL-1 bind to recombinant hsp70 and mammalian hsp70.PC (Theriault et al., 2006). Hsp70 chaperoned peptide bind to

DCs through LOX-1, and anti-LOX-1 Ab as well as LOX-1 ligand compete with hsp70 for binding to DCs. The interaction between hsp70.PC and LOX-1 promotes Ags cross-presentation on DCs (Delneste et al., 2002). The nature of adenosine phosphate moieties (ADP or ATP) associated with hsp70 affects the binding of it to scavanger receptor. Deletion mutant studies indicate that sequences in the intracellular domain of both LOX-1 and SREC-1 are dispensable for hsp70 uptake, ruling out conventional pathways of 'internalisation motif' found in the intracellular domain of many internalisation receptors. Although ADP/ATP association affects the binding of hsp70 to these receptors, no role of ADP/ATP was found in the internalization of hsp70. It was also observed that hsp70 binds to these scavanger receptors with distinct affinities (Theriault et al., 2006). Scavenger receptor class-A (SR-A), which is a potent scavenger receptor of Mϕ and DCs, serves primarily for gp96 and calreticulin recognition. gp96 internalization and peptide re-presentation are inhibited by the SR-A inhibitory ligand fucoidin. Mϕ of SR-A$^{-/-}$ mice show impaired gp96 binding and uptake (Berwin et al., 2003) confirming the role of SR-A as a gp96 receptor.

1.5.3.4 Toll-like receptors

First time Hsp60 was identified as TLR4 ligand, when recombinant hsp60 failed to show functional response on the Mϕ of C3H/HeJ mice, that carried a mutant *Tlr4* gene (Ohashi et al., 2000; Vabulas et al., 2001). It was further observed that hsp70 can also signal through TLR2 and TLR4 (Vabulas et al., 2001). Mycobacterium HSPs use diverse TLR pathways to activate pro-inflammatory signals by APCs (Bulut et al., 2005). Hsp70 stimulates IL-12 production by Mϕ, which was abrogated by introducing a double negative construct of MyD88 and TRAF6 that play the downstream signaling via TLR4. NF-κB activation was found upon hsp70 interaction with TLR2/4 (Vabulas et al., 2002). Hsp70 and Hsp90 associate with TLR4 in response to LPS (Triantafilou and Triantafilou, 2004).

It has been recently confirmed that gp96 is also an endoplasmic chaperone for cell surface TLRs in Mϕ. Despite normal development and activation by IFN-γ, TNF-α and IL-1β, Mϕ from gp96-deficient mice, failed to respond against TLR ligands, including TLR2, TLR4, TLR5, TLR7 and TLR9 ligand. Direct role of TLR-gp96 interaction in endotoxemia and *Listeria* infections and function as master chaperone for TLR mediated signal transdcution is also established (Yang et al., 2007). Hsp70 also binds to TLR2/4 (Yang et al., 2007). *Helicobacter pylori* hsp60 induces IL-8 release via a TLR2 and MAP kinase pathway in human MOs (Zhao et al., 2007). Interaction of TLR2 and TLR4 ligands with the N-terminal domain of gp96 amplifies innate and adaptive immune responses (Warger et al.,

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2006). Recently a peptidoglycan recognition protein Tag7 is shown to form a stable 1:1 complex with hsp70 (Sashchenko et al., 2004), it was also shown that cytotoxic immune cells can recognize HLA-negative but Hsp70-exposing tumor cells through Tag7 to mediate contact killing (Sashchenko et al., 2007).

1.5.3.5 CCR5

CC chemokine receptor 5 (CCR5) is a member of CC-chemokine receptor family. CCR5 has the characteristic structure of a seven transmembrane G protein-coupled receptor (GPCR), which regulates trafficking and effector functions of DCs (Balistreri et al., 2007). Recently it was shown that the microbial hsp70 binds to CCR5 in CCR5-transfected cells and in primary human cells (Mackay and Sallusto, 2006). Significant CCR5-mediated calcium mobilization was stimulated by microbial hsp70 and inhibited with TAK 779, which is a specific CCR5 antagonist. Microbial hsp70 mediated activation of the p38 MAPK and signaling pathway was also demonstrated in CCR5-transfected HEK 293 cells (Pido-Lopez et al., 2007). It was also observed that mycobacterial hsp70 induces signaling in human DCs through CCR5. Hsp70 promotes DCs aggregation; immune synapse formation between DCs and T cell and generates the effective immune response through CCR5. Mycobacterial hsp70 and the CCR5 natural agonist RANTES induce IL-6 release from DCs. The identification of CCR5 as the critical receptor for mycobacterial hsp70-medated DCs stimulation has implication for both mycobacterial infection and in the therapeutic use of mycobacterial hsp70. The cellular aggregation induced by mycobacterial hsp70 signaling through CCR5 may play an important role in the formation of granulomas, the hallmark of Mycobacterial infection (Floto et al., 2006; Whittall et al., 2006). These findings imply a novel role for CCR5 in innate immunity, in addition to its more established role as a chemoattractant receptor for adaptive immune responses.

1.5.3.6 c-type lectin like receptors (CLRs)

c-type lectin family receptors such as Dectin-1, killer cell lectin-like receptor and CD94 are expressed as hetero or homodimers on immune cells and bind to Hsp70 (Gross et al., 2003a).

Recently binding of Hsp70 was confirmed to NKG2D (typeII, CLRs), for which distinct MHC I-like molecules were known as functional ligands earlier. A minimal binding of hsp70 to other CLRs has been observed that includes DC-SIGN, CLEC-1 and CLEC-2. It was also envisaged that NKG2D may recognize surface expressed hsp70 on tumor cells and

mediate the tumor lysis (Theriault et al., 2006; Calderwood et al., 2007). Theriault, etal. had purified hsp70 from mammalian source that includes members of 'hsp70 superfamily' like HSPA1A product, heat shock protein 70 kDa protein 8, Grp78 and other hsp70 family members (Theriault et al., 2006). Given the homology between hsp70 superfamily and other HSPs family it will be interesting to observe the binding of other hsp family members to NKG2D and its functional responses on immune cells.

1.6 DCs AND ANTI-TUMOR IMMUNE RESPONSES

DCs play a crucial role in the induction of Ags-specific T-cell responses, and therefore are being used for the immunotherapy against various malignancies (Osada et al., 2006). As "nature's adjuvant," DCs have emerged as an ideal vehicle for immunization against malignancy (Hart, 1997; Jeras et al., 2005). Cancer immunity is mainly targeted to TAAs (tumor associated Ags), and a large number of TAA have been identified (Kawakami et al., 1995; Kawakami et al., 1994; Scanlan et al., 1998). Most of the TAAs are also expressed in normal tissues, and they often exhibit a poor immunogenicity (Foss, 2002). However the existence of tumor-specific Ags, which can be the target of an immune response, is also established (Renkvist et al., 2001). Investigators are constructing DCs based cellular vaccines by combining GM-CSF, co-stimulatory molecules and TAAs for tumor therapy. Paglia et al., have shown that β- galactosidase (β-gal)-transduced (in which β-gal functions as TAA) DCs can generate a long- lasting immunity against tumor challenge (Paglia et al., 1996).

A variety of immunization preparations and technologies have been developed with DCs, including whole cell tumor vaccines, tumor lysate vaccines, specific tumor Ags, tumor peptides, HSPs, HSP.peptides, DNA vaccines. Distinct tumor Ags have been investigated as possible DCs immunogens in vaccination against cancer (Sheng et al., 2005). Once stimulated, DCs can present various tumor Ags to naive T cells to initiate an immune response (Buchsel and DeMeyer, 2006).

DCs can directly trigger cancer cell death through various mechanisms. Splenic rat DCs exhibits tumoricidal activity against a wide range of tumors through NKR-P1 (Josien et al., 1997). DCs become cytotoxic against tumor cells, either spontaneously or upon IFN-γ stimulation (Chapoval et al., 2000; Trinite et al., 2005; Stary et al., 2007; Yang et al., 2001). Various maturation stimuli like CD40L, dsRNA, IFN-γ also enhance the cytotoxic function of DCs (Nicolas et al., 2007; Vidalain et al., 2001) suggesting the new role of maturation

stimuli in anti-tumor innate and adaptive responses. A pre-DC form (pDCs) links innate and adaptive immunity during microbial infection (Gill et àl., 2005). Recently it was demonstrated that microbial products (influenza virus, CpG ODN, or R848) convert plasmacytoid DCs into powerful killers against tumors through TRAIL (Chaperot et al., 2006).

In-vivo, killer DCs can play a direct effector role in anti-tumor immune responses as well as an indirect role by initiating adaptive immune responses, since killer DCs rapidly phagocytose killed tumor cells. It is possible that they use their cytolytic function to acquire tumor Ags to generate immunity (Trinite et al., 2005).

1.6.1 Clinical use of DCs

DCs have been recognized as a distinguished Ags presentation system. Recent studies indicate that Ags presentation function of DCs can be tamed against various diseases including malignancies to allow the generation of effcetive immune responses. In vaccination against tumors, such a capacity would be very desirable (Cranmer et al., 2004). The classical approach to vaccination exploits attenuated forms of pathogens to elicit an immune response, and such attenuation is now more feasible using genetic manipulation and new vectors like avipox viruses. In- vitro, DCs are the only cells that efficiently present inactivated virus (Bender et al., 1995), and therefore the efficacy of the new generation of attenuated vaccines could be improved by specific targeting to DCs.

DCs and their progenitors can be obtained from blood, BM, tumor-infiltrates, LNs, skin, umbilical cord blood or from any other suitable tissue or fluid. DCs can also be obtained by adding cytokines like GM-CSF, IL-4, IL-13 and/or TNF-α to precursors or CD34$^+$ cells. Development of easy procedures to prepare sufficient numbers of DCs paved the way for clinical trials to evaluate various DCs-based strategies in patients with malignancies. Despite many obstacles, DCs based vaccines continue to hold promise in cancer therapy (Zhong et al., 2007). Clinical grade DCs can be generated from precursor population that may be transplanted in the body to generate the desired functional responses (Campbell et al., 2005; de Vries et al., 2005; Peng et al., 2005). To avoid the differences in culture conditions and serum, serum free media have been standardized for the generation of clinical grade DCs (Napoletano et al., 2007). DCs can also be generated from precursors supplemented with autologous plasma, GM-CSF, and IL-4 in Teflon bags (closed system) that suits well for immunotherapy (Sorg et al., 2003). Given the necessity for large number of DCs, faster DCs

generation methods have also been established (Dauer et al., 2003; Jarnjak-Jankovic et al., 2007).

The ability of DCs to generate anti-tumor immune response *in-vivo* has been documented in many animal models. Most of these experiments involve isolation of DCs, loading of DCs with tumor Ags and injection of the Ags-bearing DCs into syngeneic animals as a cancer vaccine. DCs loaded with tumor lysates, tumor Ag-derived peptides, and whole proteins have been demonstrated to generate tumor-specific immune responses (Flamand et al., 1994; Mayordomo et al., 1995; Fields et al., 1998). DCs loaded with Ags have been used to induce regression of preexisting tumors (Gong et al., 1997). Imiquimod, is a TLR7 agonist and activates DCs to induces perforin, granzymeB, and TRAIL dependent apoptosis in tumors *in-vivo* , which insight a novel strategy that can be used to kill MHC I^{low} cancer cell lines (Stary et al., 2007). DCs/tumor cell fusion vaccine is another attractive approach for various types of cancer treatments and by this fusion approach, a broad spectrum of tumor Ags, including both known and unknown, are endogenously processed and presented by DCs to induce a polyclonal CTL response. Fusion strategy has been successfully used in the murine models and in preclinical human studies (Koido et al., 2007).

1.6.2 Clinical studies with DCs vaccine in anti-tumor immunotherapy

DCs pulsed with irradiated tumor cells; tumor lysates or tumor specific peptides generate anti-tumor immune response. Promising results from clinical trials in patients with malignant melanoma, multiple melanoma, breast, colon, prostrate, and cervical cancer suggest that this strategy may also function for many tumors. Clinical trials also suggest that vaccination with autologous DCs is safe, with no or little side effects.

Because of the immunosuppressive environment within a tumor mass, immunotherapy in cancer patients requires tedious vaccination protocols. DCs based approach is gaining popularity in the tumor vaccine field that immunizes cancer patients with their own DCs loaded *ex-vivo* with tumor Ags. The premise of this approach offers an efficient control over the vaccination process provided by *ex-vivo* manipulation of DCs (Gilboa, 2007).

The successful immunotherapeutic potential of DCs has been shown against acute and chronic myeloid leukemia (Westers et al., 2006; Westers et al., 2007; Yi, 2003), advanced melanoma (Guo et al., 2007), breast cancer (Pilon-Thomas et al., 2004), solid tumors (Stift et al., 2003), hepatocellular cancer (Butterfield, 2004; Sun et al., 2006), brain tumor (Parajuli and Sloan, 2004; Park et al., 2007), tounge squamous carcinoma (Wang et al., 2006), thyroid carcinoma (Stift et al., 2004), gastrointestinal carcinoma (Wu et al., 2004;Ishii et al., 2006),

ovarian cancer (Hernando et al., 2002; Santin et al., 2002), prostate cancer (Ragde et al., 2004; Thomas-Kaskel et al., 2006), renal cancer (Gitlitz et al., 2003; Ranieri et al., 2007). Vaccination with DCs transfected with mRNA-encoded folate-receptor-α cures relapsed metastatic ovarian cancer (Hernando et al., 2007). Various promising results under *in-vitro* and *in-vivo* conditions have accelerated the possibilities to cure various types of malignancy using DCs mediated approaches and phased clinical trials are under investigations (Fong and Engleman, 2000; Gilboa, 2007).

1.7 AK-5 TUMOR MODEL

AK-5 is a rat histiocytic Mφ-like tumor cell line that arose spontaneously upon i.p. injection of cell free ascitic fluid of ZAH hepatoma (Khar, 1986; Khar et al., 1990). AK-5 tumor cells grow as solid tumor when injected s.c., intradermally, or intramuscularly. Upon s.c. transplantation AK-5 cells grow as solid tumor upto 15 days, followed by spontaneous regression and healing, that exhibit highly immunogenic nature of AK-5 tumor (Bright et al., 1994), *in-vivo* AK-5 tumor regression takes place by apoptosis (Khar et al., 1995). On the other hand intraperitoneal transplantation of AK-5 tumor cells lead to ascites, resulting in 100% mortality in wistar rats (Khar et al., 1998).

AK-5 tumor-induced modulation of host immune function upregulates Th1 type cytokine response, which is involved in tumor regression (Khar et al., 1996). A single cell clone of AK-5 tumor cell was also adapted to grow *in-vitro* i.e. BC-8 (Khar and Ali, 1990). Surface molecules on fixed AK-5, and fixed BC-8 cells are highly immunogenic and activate DCs and NK cells (Alli and Khar, 2004).

AK-5 tumor cell is MHCI⁻ (Savithri and Khar, 2006), secreates TGF-β1 (Mitra and Khar, 2004) and no direct role of cytotoxic T cells has been observed during spontaneous regression of the tumor. Instead, innate immune responses have been shown to participate in the spontaneous regression of solid tumor that involves DCs, Mφ (Alli et al., 2004; Alli and Khar, 2004; Mitra et al., 2004; Bhaumik et al., 2001), NK cells (Khar et al., 1997; Mitra et al., 2003) and anti-tumor antibody produced by B cells (Khar and Anjum, 2001).

Like AK-5, many tumors are potentially immunogenic, since they show tumor-specific immune response *in-vivo*, but spontaneous regression of established tumors by endogenous immune mechanisms is a rare phenomenon, which is unique for AK-5 tumor model system. Overall, transplantable AK-5 tumor model is a simplified replication of a tumor or cancer situation that can be explored to develop a wide range of anti-tumor immune responses and therapies.

CHAPTER II

Materials And Methods

2.1 MATERIALS

2.1.1 Animals

Inbred colony of Wistar rats or Balb/c mice of both sex and 5-7 weeks old of age were used in the study. All animal experiments were done following the institutional guidelines and with the approval of the animal ethics committee.

2.1.2 Tumors and Cell lines

AK-5 tumor was maintained as ascites by injecting 5×10^6 AK-5 cells (histiocytoma) i.p. in wistar rats and solid tumors were obtained by injecting 3×10^6 AK-5 cells s.c. ZAH tumor (hepatoma) was maintained in wistar rats (i.p.). BC-8 (*in-vitro* adapted single cell clone of AK-5 tumor) was maintained *in-vitro* in IMDM supplemented with 10% FCS. For *in-vivo* experiments, tumor cells were pooled from respective host. YAC-1, MCF7, K562, A375, HeLa, J774, P19, RNK16, Rat2, F111, and PCC4 tumor cells were maintained *in-vitro* in IMDM supplemented with 10% heat inactivated FCS. Recombinant GM-CSF secreting CHO clone was a generous gift by Dr. Kris Theilemans (University of Brussels, Belgium)

2.1.3 Tissue Culture media

IMDM (Iscove's Modified Dulbecco's Medium), RPMI-1640 (Rosewell Park Memorial Institute), DMEM (Dulbecco's Modified Eagle's Medium), were purchased from Gibco BRL, Life technologies, USA. Fetal calf serum (FCS) was purchased from Gibco BRL, USA and stored at -70^0C. Penicillin, Streptomycin, Kanamycin and Gentamycin was purchased from Sigma. Cell culture media were supplemented with 12% heat inactivated FCS as complete media and pH of medium was maintained at 7.2-7.4. Trypsin-EDTA was used for detachment of cell lines and primary cells from plastic culture plates. 0.84% Ammonium chloride was used as hypotonic shock to remove RBCs from lymphocyte and BM cell preparations. Phosphate buffer saline (PBS, pH 7.2-7.4) was used for washing and passaging of cells.1-2 % Formaldehyde, 1% paraformaldehyde, and 80% methanol were used for fixation of cells for various experiments.

2.1.4 Reagents

W1400 (iNOS inhibitor), PD98059, were from Calbiochem. LPS (*E.coli* serotype 0127:B8), wortammanin, Ly294002, H7, Genistein, Okadaic acid, and polymyxin B coated agarose beads and anti-GST ab were purchased from Sigma. NF-κB specific inhibitory

peptides SN50 and SN50 M were purchased from BIOMOL. Human rIL-2(R&D) and Ovalbumin (Sigma), Murine rIL-4 (R& D), recombinant GM-CSF was purchased from R&D system. Dynal beads and detach-a-bead were purchased from Dynal, Invitrogen. 100 base pair (bp) DNA ladder (New England Biolabs, USA), Actinomycin-D (Sigma Aldrich, USA), BCIP (5-bromo-4-chloro-3-indolyl phosphate) (Sigma Aldrich, USA), Chemiluminescence Western Blotting Kit (Mouse/Rabbit) [Roche Diagnostics, GmbH (Mannheim, Germany)], DAPI (Sigma Aldrich, USA), Ficoll Hypaque (Sigma Aldrich, USA), Low molecular weight protein markers (Amersham Pharmacia), MTT (3-4,5-dimethylthiozol-2-YL 2,5-diphenyltetrazolium bromide) (Sigma Aldrich, USA), NBT (Nitro blue tetrazolium) (Sigma Aldrich),Omniscript reverse transcriptase kit (Promega, USA),Sodium Nitrite ($NaNO_2$) (Sigma Aldrich,USA),TEMED (N,N,N',N'-tetramethylethylene diamine) (Sigma Aldrich, USA),Trizol reagent (Gibco Laboratories, UK), Zymosan A particles from *S. aureus* (Sigma Aldrich,)

2.1.5 Primary and secondary antibodies

Hybridoma OX-62 ($\alpha\varepsilon$-integrin) was kindly provided by Dr M.J. Puklavec and GL-1 (CD86) by Dr Vijay Kuchroo. Anti-mouse IL-12 (c17.15, p40 subunit) was kindly provided by Dr G. Trinchieri. mAb 3.2.3 (NKR-P1) and OX-41 (Signal inhibitory regulatory protein, SIRP) were from Serotec. Anti-mouse hsp70 Ab (Stressgen). MHC II (OX-6), CD1a (FITC tagged) and mouse iNOS mAb were from BD Biosciences. Anti-ICAM1 (clone IA29) was provided by Dr. M. Miyasaka. Anti-CD8 (OX-8), Anti-TNFα and anti-IL1β were from BD Biosciences. A hybridoma secreting mAb 1A6 against NKR-P2 was raised in our laboratory using Balb/c mice. Clone 3C6 (IgM) a non-agonistic anti-NKR-P2 mAb, was used as internal negative isotype control throughout the study. Rabbit anti-mouse NFκB (p65 subunit), polyclonal anti-NKG2DAb and IκBα were from Santacruz. Anti rat/mouse/rabbit IgG either alexa-488 or alexa-594 conjugated were used as secondary Ab in accordance with primary antibodies. Alexa-conjugated antibodies were purchased from Molecular Probes. Anti-mouse iNOS was from BD Transduction Laboratory. Rabbit anti-mouse NFκB (p65 subunit), rabbit anti-mouse FasL, goat anti-mouse NKG2D and IκBα antibodies were purchased from Santacruz. Anti-human CD16, anti-CD3, anti-Ed1 was from Serotec. Antibodies for total p38, pp38, AKT, pAKT, ERK1/2 and pERK1/2 were from BD Biosciences. Similarly antibodies against MHC II, CD11c CD1a (FITC-tagged) were from BD Biosciences.

2.1.6 Radioactive isotope

Radioactive sodium chromate (Na251CrO4) was obtained from BRIT, Mumbai and had a half-life of 28 days, with a specific activity between 5-10 milli Ci/ml and was stored at –20oC. It was used to label various target cells for cytotoxicity assay. Tritiated thymidine 3(H) TdR was purchased from BRIT, Mumbai mCi/mole ($t_{1/2}$: 12 yrs) and was used to label T cell for proliferation assay.

2.1.7 Oligonucleotides

All the nucleotides used in this study were synthesized in the oligonucleotide-synthesis facility of this institute. Table 2.1 summarizes the list of primers used in this study.

2.2 METHODS

2.2.1 Generation and Isolation of DCs (BMDC, SDC and PDCs) and NK cell

BM cells were obtained from the femur of the rats. After RBC lysis (hypotonic shock with 0.84% NH_4Cl) BM cells were plated in the culture medium (IMDM, 10% heat inactivated FCS, 100 U/ml penicillin 50 μg/ml streptomycin) for 1h for the removal of resident Mφ. The non-adherent cells were collected and replated in complete medium (CM) supplemented with GM-CSF (obtained from GM-CSF secreting CHO cells, GM-CSF content >10 ng/ml and mouse recombinant IL-4 (R & D system, 5 ng/ml) for 6 days to generate iDCs. Rat BMDCs (Bone marrow derived DCs) thus obtained were OX-62, CD11c, MHCII and CD1a positive, less than 2-3% attached cells were positive for OX-41 (SIRP). Similar procedure was followed to generate mouse BMDCs. Similarly spleens were removed aseptically from experimental animals and teased in cold PBS. Total splenocytes were obtained by Ficoll-Hypaque density gradient, washed and plated for 30 min for Mφ (attached cells) elimination. The non-adherent cells were collected and splenic DCs were isolated using Dynal magnetic beads coated with OX-62 mAb (anti-CD103) as per manufacturer's description (Dynal, Chantilly, VA, USA). For phenotypic characterization both SDC and PDC were immediately fixed with 2% paraformaldehyde after magnetic bead (OX62) separation.

Total peritoneal cells were obtained by injecting cold PBS in the peritoneal cavity and resuspended in IMDM medium supplemented with 10% heat inactivated FCS, and cultured in

Table 2.1. Primers used in this study.

GAPDH	For	TGAAGGTCGGTGTGAACGCATTT
	Rev	TGATGGCATGGACTGTGGTCATG
β- Actin	For	CCGGCCAGCCAGGTCCAGA
	Rev	CAAGGCCAACCGCGAGAAGATG
iNOS	For	CACATCTGGCAGGATGAGAA
	Rev	GAAGGCGTAGCTGAACAAGG
IL-12	For	GCCCCTGGAGAAACGGTGAC
	Rev	CGCCCCTTTGCATTGGACTTC
IL-1β	For	GGCGGCATCCAGCTACGAATCTC
	Rev	CCCGGAGCGTGCAGTTCAGTG
TNF-α	For	AGCGCAGCAGAGGCACGAGAG
	Rev	GCCGCACAGTCCGCACACAC
NKR-P2	For	CTTCCTCCAGAGATGAGCAA
	Rev	TCCCCTTGTTTTACCGGACA
IFN-γ	For	ATGAGTGCTACACGCCGCGTCTTGG
	Rev	GAGTTCATTGACTTTGTGCTGG
n-Irp94	For	ACGCGTCGACAATGTCGGTGGTGGG
	Rev	CCCAAGCTTACTAGTTGTTTCATTCAT
c-Irp94	For	GGAATTCCCCAGTGCGGCCTTA
	Rev	CCGCTCGAGATCAATGTCCATCTCA
Fl-Irp94	For	CTAGCTAGCATGTCGGTGGTGGGCATA
	Rev	AAGGAAAAAAGCGGCCGCTCAATCAATGTCCAT

100 mm Petri-dishes for 1 h at 37°C. The non-adherent cells were removed by gentle washing with warm IMDM (37°C) and the adherent cells i.e. DCs and Mφ were cultured for additional 10h at 37°C, whereas most Mφs remained attached after this period, DCs became non-adherent. The non-adherent fraction was further enriched for DC with mAb OX-62 coated magnetic beads (Dynal). Using the above-mentioned protocol, we obtained about 95% pure SDCs and PDCs as assessed by morphology and phenotypic markers (OX-62, CD11c, MHCII). Peritoneal adherent Mφ were isolated by aspirating peritoneal cavity of experimental animals with chilled PBS followed by removal of floating cells after 1h, similarly adherent splenic Mφ were obtained from mononuclear lymphocytes excluding SDCs (after magnetic bead separation for DC with OX-62) by removal of floating cells after 30 min.

Splenocytes from normal rats were used for NK cell isolation using magnetic beads coated with 3.2.3 mAb as described earlier. Beads were washed extensively and the non-adherent NK cells were collected after Detach-a-bead separation. Rat PBMCs were isolated from blood (heparinzed) by Ficoll-Hypaque density centrifugation.

2.2.2 Isolation and priming of CD8$^+$T cells

Balb/c mice (8 weeks) were injected with ovaalbumin Ag (2mg/animal) for priming of CD8$^+$T cells, After 7 days total splenocytes were isolated by Ficoll-Hypaque density centrifugation. CD8$^+$T cells were isolated by a II step positive selection procedure (anti-CD3 followed by anti-CD8 coated dynal beads). Afterwards CD8$^+$T cells were incubated with rIL-2 (human rIL-2 .5ng/ml) for 48 hours and the cells were harvested for staining. Similarly rat CD8$^+$T cells were isolated by a II step isolation procedure as described above.

2.2.3 Tumor growth inhibition

Anti-tumor cytostatic activity of DCs was determined by the inhibition of DNA synthesis in target tumor cells. Briefly BMDC, SDC or PDC obtained after stimulation (*in-vivo* or *in-vitro*) were cocultured with non-adherent BC-8 tumor cells at varied experimental ratio for 24h. To estimate the DNA synthesis, cells were pulsed with [^{3}H] Thymidine (1 μCi/well) during the last 10h of incubation. [^{3}H] Thymidine incorporation was determined by measuring the radioactivity incorporated in BC-8 cells and the results are expressed as cpm for triplicate wells ± SEM.

2.2.4 Apoptosis assay

Adherent BMDC, SDC or PDC were activated with mAb1A6 for 4 h in the presence or absence of W1400 along with relevant controls and cocultured with BC-8 cells at 5:1 ratio. Induction of apoptosis in tumor cells was assessed by FACS after propidium iodide staining of fixed BC-8 cells after 24 h. (Briefly, harvested tumor cells were fixed in 80% methanol for 30min and washed with PBS. The cells were stained with propidium iodide (0.05mg/ml PI, in 0.1% sodium citrate,0.3% NP-40,0.02mg/ml RNASe for 30 min.)

2.2.5 Annexin V staining

Harvested tumor cells were washed with cold PBS and resuspended in 1X (10X Binding Buffer: 0.1 M HEPES, pH 7.4; 1.4 M NaCl; 25 mM CaCl $_2$) binding buffer. In 100 μl of the cell suspension Annexin V-FITC (5μl) was added. Suspension was gently mixed and incubated for 15 min in dark. Additional 400 μl of binding buffer to sample for dilution and sample was analysed by flow cytometry.

2.2.6 MTT Colorometric Assay

Cells plated in 96-well microtitre plates (0.1 x 10^6 cells/well), cultured in the presence of various stimulators and inhibitors at 37°C in CO_2 incubator, and were used in this assay. Cells cultured without any stimulator served as control. At the end of the incubation, the cells were washed twice with warm PBS, and 20 μl of stock MTT solution (5 mg/ml) was added in each well. The cells were allowed to incubate further for 4 h at 37°C. The plates were centrifuged at 1000 g for 5 min and the supernatants were discarded. To each well, 100 μl dimethyl sulfoxide (DMSO)-methanol (1:1) was added to dissolve the dark blue crystals formed in the cells at room temperature (RT). The absorbance was measured at 550 nm. Each assay was done in triplicates.

2.2.7 Cytotoxicity assay

Cytotoxicity assay was performed by 4 h ^{51}Cr-release assay. DCs activated NK cells were incubated with ^{51}Cr-labeled YAC-1 cells (E:T = 25:1) for 4 h. The target cells were labeled with 250μCi $Na_2Cr^{51}O_4$ at 37°C for 45 min with shaking. The cells were washed thrice with PBS to remove the free radioisotope label. ^{51}Cr released in the medium was counted in Packard Gamma counter and the percentage cytotoxicity was calculated, as (%

Cytotoxicity = 100 x Experimental cpm − Spontaneous cpm / Total cpm − Spontaneous cpm).

2.2.8 Flow cytometry

Cells were fixed in 2% paraformaldehyde for surface staining or with methanol: acetone (1:1) for intracellular staining for 20 min at 4°C. Single cell suspension of cells were prepared in PBS containing 0.5% BSA plus 0.1% NaN3, and 5% serum to block Fc and non-specific Ig binding sites. Cell suspension (100-200μl) was incubated with specific mAbs, eg iNOS, IL-12p40, MHCII, B7-2 and FITC-cIrp94 for 90 min (standardized dilution) with gentle shaking, cells were washed three times with PBS and incubated with appropriate secondary Ab for 45-60 min with gentle shaking in dark. CD1a mAb was FITC-conjugated. Stained cells were analysed on FACS (Becton Dikinson) and data was analysed by cell quest software. On analysis, scatter gating was used to avoid cell debris.

2.2.9 Immunostaining and Confocal Scanning

Adherent cells were plated on coverslips at 0.5 x 10^6 cells/coverslip, which were placed in 6-well plates, while floating cells were kept in 1.5 ml microfuge tubes at the same concentration. Cells were fixed in 2 % paraformaldehyde for 30 min at RT, after which they were washed in PBS 3 times. The rest of the steps were carried out in PBS. The cells were blocked with 1% BSA for 1 h at RT or at 4°C overnight. 200 μl of primary Ab per 10^6 cells was added on the coverslip or into the tubes and incubated at RT for 2 h, after which the ᵥ excess Ab was washed off and the cells washed 3 times with buffer. Attached cells were washed by adding buffer to the well and shaking, while floating cells were suspended in the buffer, tubes inverted several times and then centrifuged at 800 g for 10 min to obtain the cell pellet. The cells were then incubated with appropriately diluted secondary Ab for 30-45 min, after which the cells were again washed 3 times with buffer. The cells on coverslips were finally mounted with Antifade reagent (Molecular Probes, USA) on glass slides and viewed under the fluorescence microscope.

Isolated SDCs and BMDCs were plated on coverslips, the cells were fixed with 2% paraformaldehyde and probed with goat anti-mouse NKG2D polyclonal Ab (20μg/ml) and mouse CD11c mAb for 12h at 4°C, after washing the respective primary antibodies the cells were probed with appropriate secondary Ab. The scanning was performed on LSM Carl Ziess confocal microscope.

BMDCs were cultured and stimulated on coverslips. After fixation with 2% para formaldehyde for surface staining and methanol: acetone for intracellular staining, cells were stained with primary mAb for 90 min followed by appropriate fluorochrome conjugated secondary Ab for 45 min. FITC-cIrp94 (1mg/ml) was incubated with DCs for 90 min. followed by washing. Immunostained cells were observed using confocal laser scanning microscope for mAb1A6, NF-κB and FITC-cIrp94 staining. AK-5 tumor cells were fixed in 2% paraformaldehyde (5min) for surface staining or with 80% methanol for intracellular staining and probed with mAb2F4 (90min) and the secondary Ab (30 min) and scanned on confocal microscope.

2.2.10 Estimation of NO

DCs (BMDC, SDC or PDC) were incubated at 2×10^5 cells/well (triplicate) in 96 well plates along with different activators in IMDM (Invitrogen) supplemented with 10% FCS for indicated time. Cell free culture supernatants were recovered after incubation and NO content was measured with Griess reagent. The absorbance at 540 nm was measured using ELISA reader (Molecular devices, Spectra Max 190). Nitrite content was quantified from standard curve generated using sodium nitrite. Nitrite in all samples was measured by the Greiss reaction.

The Greiss reagent consists of one part 0.1 % naphthylethylenediamine hydrochloride in water plus one part 1 % sulfanilamide in 5 % phosphoric acid (H_3PO_4). Each of the aliquots collected was analyzed for nitrite by mixing 100 μl Greiss reagent with 100 μl of each sample in a microtitre plate, and measuring the absorbance at 550 nm. Standard curve for nitrite was generated using $NaNO_2$, which had a regression coefficient of 0.9945 and regression equation, $y = 0.0152x + 0.0274$.

2.2.11 Isolation of total RNA

Total RNA was extracted from cells using Trizol reagent, as per the manufacturer's instructions. Briefly, Trizol reagent was added to the cells at 1 ml Trizol per $5-10 \times 10^6$ cells in a 1.5 ml microfuge tube, and pipetting several times in the reagent lysed the cells. The solution was then vortexed for 45 sec to shear genomic DNA, after which the tube was kept at RT for 5 min. 0.2 ml chloroform per ml Trizol used was added, and the tube was vigorously shaken for 15 sec, after capping, to mix the 2 layers of solution. This was incubated at RT for 2-3 min after which the tube was centrifuged at 12,000 g for 15 min at 4°C for phase-separation. The lower pink phase was that of phenol-chloroform containing

DNA, the white interphase consisted of protein, while the upper colourless aqueous phase consisted of RNA and comprised 60 % volume of the total solution. The upper phase was carefully collected and transferred into a fresh tube to which 0.5 ml isopropanol per ml Trizol used was added to precipitate the RNA. The tube was incubated for 10 min at RT and then centrifuged at 12,000 g for 10 min at 4°C to obtain a white RNA pellet. The supernatant was discarded and the pellet washed with 75 % ethanol (1 ml per ml Trizol used) by suspending in ethanol and centrifuging at 7,500 g for 5 min at 4°C. The supernatant was discarded and the pellet was air-dried, after which it was dissolved in autoclaved milliQ water by passing the solution through a pipette several times, and then incubating for 10 min at 55°C. The RNA was either used immediately or stored at 1 μg/μl concentration in 100 % formamide at −70°C.

2.2.12 Quantification of RNA and DNA

Nucleic acids were estimated by measuring the absorbance at 260 nm (Sambrook et al, 1989). An absorbance of 1 approximately corresponds to 40 μg ml^{-1} of RNA and 33 μg ml^{-1} of single-stranded oligomers. Purity of RNA was ascertained by taking the A_{260}/A_{280} ratio. A ratio of 1.8 corresponded to a pure preparation of nucleic acids.

2.2.13 Agarose gel electrophoresis

DNA-loading dye (0.25 % bromophenol blue, 0.25 % xylene cyanol, 30 % glycerol) was added to the cDNA samples. Based on the size of the cDNAs, either 1 % or 2 % agarose gel was cast in 1 X TAE (40 mM Tris acetate, 1 mM EDTA, pH8), containing 0.5 μg/ml ethidium bromide. The gel was electrophoresed in 1 X TAE buffer at a constant voltage of 5 V/cm. 100 bp DNA ladder marker was loaded alongside for estimating the sizes of the DNA fragments. The cDNA stained with ethidium bromide was visualized in a UV transilluminator.

2.2.14 Reverse-transcription (RT) and Polymerase-chain reaction (PCR)

RNA was prepared using 'Trizol' reagent and reverse transcription was carried out with random hexamers, using the kit from Promega, as per the manufacturer's instructions. Reverse transcription was carried out in a final concentration of 5 mM MgCl$_2$, 1 mM each of dNTPs, 2.5 μM random hexamers and 2 μg total RNA. A master mix was prepared (20 μl per reaction) and final volume was made up with milliQ water. Reverse transcription was

performed at 42°C for 15 min, denaturation and inactivation of the reverse transcription enzyme at 94°C for 5 min, followed by incubation at 4°C for 5 min.

1-2 µl of the reverse transcription product was used as template in subsequent PCRs with forward and reverse primers. The following conditions were used for PCR – denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 60 sec. After 35 cycles, a final extension was done at 72°C for 5 min. The amplified products (10 µl) were run with 100 bp ladder markers on 1.5-2 % agarose gel at 5 V/cm.

In order to determine the relative RNA levels, primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were also added in each PCR tube to serve as a control for each reaction. GAPDH was amplified for 20 cycles by adding the primers after 15 cycles of the PCR reaction. The levels of GAPDH in each tube were equalized and the levels of the other PCR products were accordingly adjusted.

Total cellular RNA was isolated using Trizol reagent. cDNA was prepared using oligo(dT) 12-18 primer and AMV reverse Transcriptase (Promega). Semiquantitative RT-PCR was carried out using specific primers for iNOS, IL-12, TNF-α, IFN-γ, IL-1β along with GAPDH primers.

2.2.15 Protein Analysis

2.2.15.1 Estimation of protein concentration

2.2.15.1.1 Bradford estimation

Protein estimation was done using Bio-Rad protein assay kit (Bradford method). The concentrated dye was diluted with four volumes of distilled water. 25 µl of sample was added to 100 µl of the diluted dye reagent. The reaction mixture was mixed thoroughly and absorbance was measured at 590 nm. Different concentrations of BSA were used as standards to make a calibration curve with a regression coefficient of 0.9995 and regression equation, y = 0.0004x – 0.0001.

2.2.15.1.2 TCA method of protein estimation

TCA method is a fast assay for cellular proteins solubilized in Lammeli Buffer. This assay is based on the measure of the induced turbidity at 570nm that follows the addition of trichloroacetic acid (TCA) to a final concentration of 24%. The protein samples were solubilized in Laemmli sample buffer [62.5 mM Tris-HCl, pH 6.8, 2.35 % SDS, 100 mM DTT, 10 % glycerol, 1 mM EDTA, 0.001 % bromophenol blue], boiled for 5 min, and centrifuged in a microfuge at 10,000 g for 5 min. Samples (150 μl) were added to a microtitre plate, 100 μl of 60% TCA (w/v, filtered) was added and the turbidity was measured at 570 nm in a microplate reader after 20 min incubation at RT. Turbidity of different concentrations of BSA was used as a standard to make a calibration curve.

2.2.15.2 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Solutions:

1. Acrylamide stock: 29 % (w/v) acrylamide and 1 % N,N'-methylene bisacrylamide.

2. Stacking buffer: 1.0 M Tris.Cl, pH 6.8.

3. Resolving gel buffer: 1.5 M Tris.Cl, pH 8.8.

4. SDS stock: 10 % (w/v) solution.

5. Ammonium persulphate (APS) stock: 10 % (w/v) solution, freshly made.

6. Gel running buffer (1X): 25 mM Tris, pH 8.3, 250 mM Glycine and 0.1 % SDS.

7. Sample loading buffer (4X): 200 mM Tris.Cl, pH 6.8, 8 % SDS, 40 % Glycerol,

400 mM β-mercaptoethanol (β-ME), 0.4 % Bromophenol blue dye.

Gels of 1.5 mm thickness were cast in a Hoefer Mighty gel apparatus. Resolving gel of 12 % (10 ml) was made by mixing 4.2 ml of 30 % acrylamide stock, 3.1 ml water, 2.5 ml of 1.5 M Tris.Cl, pH 8.8, and 0.1 ml of 10 % SDS. Stacking gel (2 ml) was made by mixing 0.33 ml of 30 % acrylamide, 1.4 ml of water, 0.25 ml of 1 M Tris.Cl, pH 6.8, and 0.02 ml of 10 % SDS. Gels were polymerized by addition of TEMED and APS (1/100th volume of gel mix). Sample dye (4X) was added to the protein samples, which were then boiled for 5 min, cooled by plunging into ice and loaded. The gel was run at constant current of 20 mA till the dye front crossed the stacking gel, after which the current was increased to 40 mA.

2.2.15.3 Immunoblotting

The protein samples separated on SDS-PAGE were transferred to nitrocellulose membrane electrophoretically. The gel and membrane were wetted with transfer buffer (39 mM Glycine, 48 mM Tris base, 0.0375 % SDS and 20 % methanol). The gel was placed in

contact with the membrane and sandwiched between graphite plate electrodes, with the membrane facing the anode. The transfer was done for 1.5 h, using a current of 0.65 mA/cm^2.

After the transfer, membranes were blocked in 3 % BSA in PBS with 0.1 % Tween-20 overnight at 4°C or for 1 h at RT. The blot was incubated with primary Ab for 1 h in binding buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05 % Tween-20), washed for 15 min with 3 changes, using wash buffer (10 mM Tris-HCl, pH 7.5, 300 mM NaCl, 0.1% Tween-20), and incubated with alkaline phosphatase- or peroxidase-conjugated secondary Ab for 1 h, and washed for 15 min with 3 changes. The proteins incubated with alkaline phosphatase-conjugated Ab were visualized by using AP buffer (100 mM NaCl, 5 mM MgCl$_2$, 100 mM Tris-Cl, pH 9.5), containing 0.005 % BCIP and 0.01 % NBT. The proteins to be detected by peroxidase-conjugated Ab were visualized by using ECL kit. The reagents A and B were mixed in the ratio 100:1 and the blot was treated with this solution at a concentration of 0.1 ml/ cm^2 for 1 min in the dark, and exposed to X-ray film for 1-10 min, after which the film was developed.

BMDCs were stimulated with mAb1A6 or LPS or other stimulant, for indicated time and lysed in Laemelli buffer. Lysates were resolved on 10% SDS-PAGE, the proteins were transferred to nitrocellulose followed by immunoblotting with anti-mouse iNOS mAb, and equal loading was confirmed by amplification of β-actin signal. Similarly immunoblotting was performed with pp38, pAKT and pERK1/2 and equal loading was confirmed by total p38, AKT and ERK1/2 specific mAbs. Total cellular extract was used to detect Iκ-Bα with polyclonal Ab and equal loading was checked with β-actin. Nuclear extract from the stimulated cells was probed with polyclonal NFκB Ab and the equal loading was confirmed by Ponceau S stain.

2.2.16 cDNA expression library construction and screening

Poly A$^+$ RNA was isolated from AK-5 tumor cells using Trizol reagent (Invitrogen). The cDNA expression library from AK-5 cells was constructed using ZAP-cDNA synthesis kit (Stratagene) as per manufacturer's protocol. The library was titrated and appropriate dilution was plated on LB plate along with the host strain XL1 Blue MRF, to obtain well-separated plaques. The plaques were immobilized onto nitrocellulose membrane on pre wet with IPTG (Sigma Chemical Co.). Replica blots were obtained to maintain negative controls during screening. The screening protocol was based on the far-western principle, wherein the blots were incubated with soluble recombinant NKR-P2-GST fusion protein or with GST alone overnight at 4^{0}C, followed by washing and incubation with anti-GST Ab and

appropriate secondary Ab conjugated with alkaline phosphatase. The signal was developed using NBT/BCIP (Sigma) chromogenic substrate. The blots were aligned with plaques and positive plaques were cored out and used for subsequent secondary and tertiary screening. Finally, individual positive plaques from enriched plates were processed for obtaining the insert encoding the interacting protein. The insert was sequenced from multiple independent plaques and the sequence identity was obtained using NCBI blast software.

2.2.17 GST pull down assay

Soluble NKR-P2-GST fusion protein was prepared. The soluble recombinant NKR-P2-GST fusion protein and GST alone were immobilized on glutathione agarose beads. Soluble recombinant Irp94 protein was incubated with Ni-NTA agarose in buffer containing 50mM Tris-Cl pH 8, 300mM NaCl, 20mM imidazole at 4^0C for 4 h. To this complex NKR-P2 GST fusion protein or GST alone was added in an equimolar ratio and incubated for 4 h at 4^0C. At the end of incubation, the beads were washed five times with PBS and the bound proteins were released in Laemmli sample buffer (2X), boiled for 5 min, and resolved on 10% SDS PAGE. The gels were silver-stained to see the pull down products.

2.2.18 Generation of recombinant Irp94 proteins, anti-Irp94 antibodies and FITC-conjugation of cIrp94

The recombinant Ischemia responsive protein constructs, Irp94 full length (1-840), Irp94 C-terminus (493-840) and Irp94 N-terminus (1-175) were cloned in pET series vectors. Full length Irp94 (Vector pET28; sites NheI and NotI), C-terminus Irp94 (Vector pET28; sites SalI and HindIII), N-terminus Irp94 (Vector pET21 EcoRI and XhoI) The recombinant protein namely C-terminus subdoamin, cIrp94 (aa 493-840) and N-terminus ATPase, nIrp94 (aa 1-175) were overexpressed in *E. coli* BL21 DE3. Full-length Irp94 (aa1-840) was expressed in Rosetta DE3. Cloned inserts were verified by DNA sequencing. Ni-NTA agarose was used as the affinity matrix, The cell lysate was prepared in 10 ml lysis buffer (50mM Tris-Cl, pH 8, 300mM NaCl, 10 mM Imidazole, 1μg/ml leupeptine, Aprotinin, Pepstatin, 0.15mM PMSF) and the washing was carried out with 50mM Tris-Cl, pH8, 300mM NaCl, 20mM imidazole. Bound proteins were eluted with 50mM Tris-Cl, pH8, 300mM NaCl, 259mM Imidiazole. All the three proteins were expressed as his-tagged fusion proteins, and were purified by using Ni-NTA agarose column, and the identity of each protein was confirmed by MALDI-TOF analysis. Extensive washing before protein elution, was adopted as an essential practice to diminish chances of LPS contamination during

purification. The protein preparations were treated with polymyxin B- coated agarose beads (Sigma) to remove LPS. c-Irp94 was injected s.c. in mouse, antisera (polyclonal anti-Irp94) were collected and used for immunostaining. The mAb 2F4 against Irp94 was generated using the recombinant c-Irp94 protein. The specificity of mAb 2F4 was checked by immunostaining of AK-5 cells and with full-length Irp94 cDNA transfected CHO cells. Full length Irp94 transfected and Hsp70 (Hsp70Cκ) transfected (lipofectamine, Invitrogen) CHO and parental control cells after fixation were stained with NKR-P2-GST fusion protein, followed by detection with anti-GST and anti-rat Ig-FITC antibodies and the surface staining was analysed by FACS. Similarly, nontrypsinized full length NKR-P2 cDNA transfected CHO cells and parental CHO cells were stained with FITC-cIrp and analysed by FACS.

2.2.19 ELIFA (Cytokine Assay)

IL-12, TNF-α, IFN-γ and IL-1β levels in culture supernatants were measured by ELIFA (Enzyme linked immunofiltration assay, Pierce, USA). Briefly, culture supernatants were filtered through nitrocellulose membrane, which traps the cytokine/proteins specifically, followed by blocking with BSA (3%) and capturing with appropriate mAbs. HRPO clubbed signal was infiltered with color sensitive substrate in 96 well and read at 490 nm along with negative and positive controls.

2.2.20 MLR Assay

For the MLR assay, T lymphocytes were enriched by nylon wool followed by anti-CD3 mAb coated Dynal beads. The purity of T cells was >95% as assessed by FACS using anti-CD3 mAb. After 24 h stimulation with 1A6, attached experimental BMDCs (blocked with rNKR-P2 protein) were washed and cultured at 1:20 ratio with autologous T lymphocytes for 3 days, and further treated with 1A6. Proliferation was measured after 3 days. At 18 h before termination, 1.0 μCi [³H]-thymidine was added per well. Cells were harvested and the incorporated radioactivity was measured in a β-counter.

For other experiments, after 24 h stimulation with nIrp94, cIrp94, and full-length Irp94 proteins, attached BMDCs were washed and cultured with autologous T lymphocytes at an appropriate ratio for 3 days in conjunction with fractions of the proteins, which had been used for DC treatment. Proliferation was measured after 3 days. At 18 h before termination, 0.5 μCi of [³H]-thymidine was added per well. Cells were harvested and the incorporated radioactivity was measured in a β-counter.

2.2.21 Quantitation of Endocytosis

Zymosan particles of *Staphylococcus aureus* were labeled with fluorescein isothiocyanate (FITC), following the same procedure as labeling of antibodies [Harlow and Lane, 1988]. Briefly, the zymosan particles were suspended in 0.1 M sodium carbonate (pH 9.0) at 2-mg/ml concentrations. FITC was dissolved in DMSO at 1 mg/ml concentration. For each ml of the protein solution, 50 µl of the dye solution was added very slowly while the protein solution was gently but continuously stirred. The reaction mixture was left in the dark for 8 h at 4°C. NH$_4$Cl was added to a final concentration of 50 mM and further 2 h incubation was done at 4°C. Glycerol was added to a final concentration of 5%. The particles were then washed several times in PBS till the background colour was removed. For fluorescein coupling, the ratio of fluorescein to protein was estimated by measuring the absorbance at 495 nm and 280 nm. The ratio should be between 0.3 and 1.0, and in our case, it was 0.9.

BMDC and SDC were stimulated with mAb1A6 for 24 h, washed, resuspended in complete media and incubated with zymosan-FITC (1 mg/ml) for 1 h at 37°C. Uptake was stopped by washing the cells with chilled PBS containing 0.01% NaN$_3$. Cells were washed four times and analyzed with FACS. Surface-binding values obtained by incubating cells at 4°C were subtracted from the values measured at 37°C.

2.2.22 LAL (Limulus amebocyte lysate) assay and Endotoxin removal

LAL assay was performed (Biowhittakar) within the sensitivity level of 0.125 EU. Endotoxin levels were always below the permissible limits (10 pg/mg). LAL assay is a qualitative/quantitative test for gram-negative bacterial endotoxin. Limulus amebocyte lysate as supplied was reconstituted with LAL reagent water and then mixed in equal parts with the test solution. After incubation, and in the presence of endotoxin, gelatination occurs; in the absence of endotoxin, gelation does not occur. LAL assay was performed as per CAMBREX BIOSCIENCES WALKERSVILLE, Inc (BIOWHITTAKER, A Cambrex company) protocol. Briefly, each assay includes a series of two-fold dilution of endotoxin standard, which brackets the labeled lysatae sensitivity, dilution of the test sample and LAL reagent water to serve as negative control. Carefully transfer 0.10 ml of standard, sample, or water into the appropriate 10x 75mm reaction tube. Then 0.10 ml of lysate was added to tubes. Immediately after addition the contents were mixed gently and the tubes were placed at 37°C on dry water bath. After 2h of incubation each tubes was examined for gelation. A postive reaction was indicated by firm gelation that remains intact momentarily when the tube was

inverted at 180^0. Dilution series of range 0.5EU/ml to 0.03 EU/ml was used to perform the assay.

Polymyxin B-agarose (1 ml; Sigma) was washed twice with and resuspended in 0.5 ml of low-endotoxin water (Biowhittaker). Equal volumes (0.5 ml) of recombinant proteins, or Ab suspension were mixed with washed polymyxin B-agarose at 4°C, and the polymyxin B-agarose was collected by centrifugation. The supernatant was removed for endotoxin activity (LAL assay), or protein assays (conducted as described above); this process was repeated two times to remove endotoxin from desirable reagents.

2.2.23 Generation of antibodies

The polyclonal serum against cIrp94 was obtained by injecting bacterial overexpressed proteins as the source of Ag. Purified protein was injected as Ag dose of 100 μg per animal . A group of three 5-7 week old Balb/c mice were injected with Ag mixed with equal volume of Fruend's adjuvant. The injection was repeated after 15 days with incomplete Fruend's adjuvant. Afterward booster was administrated and serum was collected from mice, 15 days after booster dose, when the Ab titre reached maximum. Collected serum (retroorbital bleeding) was kept at room temeperature for 2h for clotting and sera (supernatant) were colleted. Cetrifugation was done to remove remaining clot from serum. The serum was tested for its specificity by immunofluorescence and western blotting. Ag boosters were administrated to animals and the sera were collected and stored for further experiments.

A mAb against cIrp94 was raised. 5-7 week old Balb/c mice were injected with cIrp94 and Fruend's adjuvant. After the optimum humoral response, screening was performed by immunofluorescence on AK-5 tumor cells and by ELIFA with recombinant cIrp94. 5 days before fusion, mice were given booster dose with Ags. The mouse mylemoma cells (Sp2/O) were used as the fusion partner. Splenocytes from Ags injected animals were obtained and cell fusion (myeloma- splenocytes) was performed in serum free media. The cell suspension was pooled and centrifuged at 1000rpm for 10 min. 50% PEG solution was added while resuspension of cells. 10 ml of serum free media was added to the cell suspension. Cells were resuspended in 10 ml of media with 20% FCS, HAT (100X: 10mM hypoxanthine, 1.6mM thymidine and 0.04 mM aminopterine). Cells were transferred to 100 ml of HAT media and 100μl of cell suspension was distributed into 96 well flat bottom plates and kept at 37^0C in CO_2 incubator. Screening was performed 10-15 days after fusion. Briefly, 50μl of supernatant was collected from each well and screening was performed on BC-8

tumor cells. The clones were graded based on the binding strength by immunofluorescence and ELIFA. Single cell clone were obtained by limiting dilution method. Ab was purified by ammonium sulphate precipitation or by Protein A sepharose column.

2.2.24 Precipitaion of antibody

Hybridoma culture supernatant was precipitated by ammonium sulphate crystal to a final concentration of 45% with stirring at 4^0C. After centrifugation (10,000 rpm for 30 min) the precipitate was suspended in PBS. Ab solution was dialysed against cold PBS for 18h. Purified Ab was recovered and stored at -20^0C.

2.2.25 Fluorescination of protein

cIrp94 was conjugated to FITC, as described (Antibodies, A Laboratory manual, Cold Spring Harbor Laboratory.) Briefly, for each ml of protein solution (2 mg/ml), 50 µl of FITC dye was added. After 8 h incubation in dark at 4°C, unbound dye from conjugate complex was removed by gel filtration (Sephadex G-25). FITC-cIrp94 protein integrity was further checked on SDS-PAGE and was found to be unaffected with FITC-coupling protocol. The molar ratio of absorbance for FITC-cIrp94 was 0.7 (optimal range 0.3 – 1.4).

2.2.26 Cell Aggregation Assay

Non-adherent BMDCs and SDCs were incubated with isotype control mAb and mAb1A6 for 2 h and photographed at different fields to score extent of homotypic clustering.

2.2.27 AK-5, ZAH Tumor Growth Studies

Male Wistar rats (4-6 weeks/5 animals per group) were challenged with tumor cells s.c. (3 x 10^6 cells/animal) and were given 9 Ab injections (1A6 1mg/ml, i.p. or s.c.) on alternate days. Isotype matched control Ab was used in the control group. Tumor growth was monitored on different days. Wistar rats were given 12 mAb1A6 injections for AK-5 ascites, 6 mAb1A6 injections for ZAH-ascites. Animals were routinely monitored for host death and tumor growth.

2.2.28 Tumor size measurement

The tumor size was measured, using Vernier caliper, taking two diameters of the solid tumor at right angles of each other and calculating the volume of the tumor according to the following formula:

Tumor size= 4/3 π a^2b, where a=shortest diameter and b= longest diameter.

CHAPTER III

Cross-linking a mAb To NKR-P2 On Dendritic Cells Induces Their Activation And Maturation Leading To Enhanced Anti-Tumor Immune Response

3.1 INTRODUCTION

The coordination of an efficient anti-tumor immune response requires the recognition of tumors and subsequent induction of innate and adaptive immune responses, which are critical for the successful eradication of tumors. DCs are pivotal part of immune system by virtue of their high Ag-presentation functions.

DCs are found at various anatomical locations as different subsets and maintain immunological homeostasis by sampling self-Ags (Banchereau and Steinman, 1998). DCs rapidly infiltrate in tumor mass (Bergeron et al., 2006; Preynat-Seauve et al., 2007; Stary et al., 2007). Clinically, an increased number of tumor-infiltrating DCs have been reported to correlate with a better prognosis in cancer patients (Kobayashi et al., 2007; Lesimple et al., 2006; Thomas-Kaskel et al., 2007; Osada et al., 2004) and DCs based immunotherapy is highly effective for the inhibition of tumor metastasis or recurrence (Chang and Dhodapkar, 2003; Lim et al., 2007).

Large number of animal and human studies has shown that Ag-loaded DCs lead to anti-tumor immune responses with tumor regression and rejection (Schott, 2006). DCs exhibit anti-tumor function by their specific T cell regulatory capacity (Lanzavecchia and Sallusto, 2001), which is generated by the presentation of tumor Ags upon DCs maturation (Nouri-Shirazi et al., 2000; van den Broeke et al., 2003). Earlier evidences have also shown that DCs mediated anti-tumor responses are associated with its 'adjuvant function' by stimulating cytotoxic T cell responses (Mayordomo et al., 1995; Zhang et al., 2007b) and Th1-mediated tumor immunity (Hokey et al., 2005).

Recent reports have revealed that DCs can directly induce tumor cell death (Trinite et al., 2005). Overwhelming evidences have accumulated in favour of DCs mediated T cell independent anti-tumor immune response to execute tumor (Lee et al., 2007). Human umbilical cord blood DCs kill tumor cells without damaging normal hematological progenitors (Shi et al., 2005). Tumoricidal potential of blood DCs have been recently demonstrated (Schmitz et al., 2005). DCs induce the death of human papilloma virus-transformed keratinocytes but not of normal keratinocytes, suggesting their killing specificity against pre-neoplastic cells (Hubert et al., 2001). Human DCs mediated cytotoxicity against breast carcinoma is also reported (Manna and Mohanakumar, 2002). In-vitro growth inhibition of a broad range of tumor cell lines by activated human DCs has been observed (Chapoval et al., 2000). Mouse and human DCs also become cytotoxic against tumor cells, either spontaneously or after activation with IFN-γ (Schmitz et al., 2005; Trinite et al., 2005; Yang et al., 2001). Nicolas et.al. have recently reported that rat

DCs trigger NO mediated tumor cell death upon LPS or IFN-γ treatment (Nicolas et al., 2007). TNFα release has also been proposed as a killing factor against TNF-sensitive cancer cells by DCs (Chapoval et al., 2000; Vanderheyde et al., 2001).

Direct tumor recognition and killing through TRAIL, FAS-ligand, lymphotoxin $\alpha_1\beta_2$, and NKR-P1 receptors have been reported (Lu et al., 2002; Trinite et al., 2000). Human MOs-derived DCs exhibited potent lytic activity towards various human tumor cell lines of distinct origin (Janjic et al., 2002; Yang et al., 2001). Recently Stary, G., etal have also reported that DCs loaded with cytotoxic proteins (perforin, granzyme), act as weapons of tumor mass destruction *in-vivo;* they have demonstrated that skin tumors (basal cell carcinomas) regress when smeared with Imiquimod, a drug that activates DCs through TLRs (Stary et al., 2007). Recent reports also demonstrate that 'like NK cells' DCs kill tumors that lack MHCI. It has been demonstrated that 'like T cells' pDCs specifically destroy tumor cells *in-vitro.* Thus varied target specificity implies that DCs have intrinsic capacity to kill all kinds of tumor cells (Bashyam, 2007; Stary et al., 2007).

Rat cytotoxic DCs express NKR-P2/NKG2D activating receptor (Alli et al., 2004). NKR-P2 is an ortholog of human and mouse NKG2D. NKG2D is a 24.4-kDa disulfide linked homodimer belonging to type-II c-type lectin like receptor also expressed on NK cells (Alli et al., 2004) CD4$^+$, CD8$^+$αβ, γδ$^+$T cells (Coudert and Held, 2006). Transcript of NKR-P2 has been shown on rat CD4$^+$/CD8$^+$ MOs (monocytes) and Mφ (Baba et al., 2006). Recently NKG2D was also reported on a cytotoxic subset of mouse DCs (Taieb et al., 2006, Chan et al., 2006), human myeloblastic KG1a cells (Guilloton et al., 2005), that recognizes various stress inducible ligands on tumor cells and generates effective immune response (Raulet, 2003).

DCs maturation is characterized by the modulation of distinct surface receptors and differential capacity of Ag processing and presentation (Banchereau and Steinman, 1998). Activation and maturation associated fine reprogramming of DCs against neoplastic cells is also exerted through apoptotic and necrotic cells tumor cells (Demaria et al., 2005, Sauter et al., 2000).

Enhancement of immune response by cross-linking specific receptors on immune cells has emerged as a potential therapeutic tool in cancer treatment (von Mehren et al., 2003). Antibody mediated activation and maturation of DCs have been reported by cross-linking Fcγ (Kalergis and Ravetch, 2002), CD32 (Banki et al., 2003; Corinti et al., 1999; Fedele et al., 2004), CD38 (Fedele et al., 2004), CD40 (Caux et al., 1994), CD43 (Corinti et al., 1999), CD95 (Guo et al., 2003; Rescigno et al., 2000), MHC II (Andreae et al., 2002) and

B7-DC receptors (Blocki et al., 2006). Previously, it was reported that splenic DCs undergo maturation after coculture with fixed AK-5 tumor cells (Alli and Khar, 2004) and a direct involvement of NKR-P2 in the induction of DCs mediated anti-tumor immune response was shown (Alli et al., 2004). These observations suggest that stimuli dependent concerted anti-tumor responses can be utilized to comprise DCs as potential immunotherapeutic agents.

In the present study we show the expression of NKR-P2/NKG2D on DCs isolated from various anatomical locations. We demonstrate that cross-linking of NKR-P2 with an agonistic NKR-P2 mAb (1A6) on rat DCs provides a strong stimulatory signal, which in turn enhances apoptosis in tumor targets through NO and also induces cytostatic effect on tumor growth. 1A6 mediated DCs activation is dependent on p38, PI3K, ERK1/2, PKC (protein kinase C), NF-κB translocation and Ca^{++} mobilization, suggesting the involvement of MAP-kinase pathway in NKR-P2 mediated DCs activation. Interestingly, 1A6 also transmits the effective maturation signal in DCs, which is characterized by enhanced MHC II, B7-2, CD1a expression, Ag presentation function and reduced endocytic capacity of DCs. Besides, 1A6 also induces DCs to secrete Th1 immunoregulatory cytokines.

AK-5 tumor is a highly immunogenic rat histiocytoma that regresses spontaneously upon s.c. transplantation in syngenic animals, whereas it kills 100% hosts upon i.p. transplantation (Khar et al., 1998). We show that 1A6 administration *in-vivo* induces activation and maturation of DCs, resulting in faster regression of AK-5 tumor (s.c. and i.p.). The present study demonstrates the stimulatory potential of anti-NKR-P2 mAb and mechanism of NKG2D/NKR-P2 mediated tumor cell death, which could be utilized to enhance the therapeutic capability of DCs for successful treatment of cancer.

3.2 RESULTS

3.2.1 Expression of NKR-P2/NKG2D on DCs

First we examined the phenotypic profile of freshly isolated splenic DCs (SDCs) and peritoneal DCs (PDCs) to assess the purity of the cell population with specific antibodies against αε-integrin, MHC II, CD11c, ICAM-I, B7-2, which are the markers of DCs; no contamination of NK cells ($CD16^+$), or Mφ ($ED1^+$) were found in DCs preparations **(Fig. 3.1A).**

NKR-P2/NKG2D expressing rat NK cells, RNK-16 cells, CD8$^+$ T cells and Bone marrow derived DCs (BMDCs) were stained with mAb1A6, which showed expression of NKR-P2 on rat BMDCs (**Fig 3.1B**). Similarly, mouse NK cells, activated CD8$^+$T cells and mouse BMDCs were stained with mAb1A6 and polyclonal anti-NKG2D Ab (**Fig 3.1C**). NKR-P2/NKG2D expression on rat BMDCs and SDCs was also confirmed by staining of CD11c$^+$ DCs (**Fig3.2A**). We also determined the proportion of NKR-P2 expressing DCs, isolated from spleen, peritoneal cavity and BMDCs. αε-integrin positive BMDCs, PDCs and SDCs were positive for NKR-P2, which was determined after staining with mAb1A6 (**Fig. 3.2B**).

3.2.2 Activation of DCs with mAb 1A6 *in- vitro*

In order to check the activation of DCs from different sites with agonistic anti-NKR-P2 mAb (1A6), pure populations of BMDCs, PDCs and SDCs were incubated with Isotype control Ab, mAb1A6, fixed BC-8 tumor cells and LPS for 24 h. NO produced in response to NKR-P2 cross-linking from BMDCs, PDCs and SDCs was estimated. About 6~7 fold increase in NO levels was observed with BMDCs and PDCs, whereas ~2 fold increase was detected from SDCs *in-vitro*. Coculture of fixed BC-8 tumor cells with DCs (25:1) and LPS (1μg/ml) stimulation was used as positive controls in the assays. In parallel assays peritoneal Mφs do not show activation with similar stimuli except LPS (**Fig. 3.3A**). We also determined if *in-vivo* administration (i.p.) of mAb1A6 induced the activation of PDCs and SDCs. PDCs and SDCs were harvested after 36 h of 1A6 injection and treated again with mAb1A6 as second dose of activator or left untreated for 24 h. About 6-fold increase in NO production was observed from 1A6-injected PDCs, which increased further upon second dose of 1A6 stimulation *in-vitro*. However, SDCs isolated from 1A6 injected animals produced ~2.5 fold more NO in comparison to control, whereas second dose of 1A6 (*in-vitro*) to SDCs further enhanced the NO secretion significantly. LPS was also used as second activator (**Fig. 3.3 B, C**).

We have also estimated tumoristatic potential of 1A6 activated PDCs and SDCs. PDCs and SDCs from 1A6-injected rats were able to suppress growth of BC-8 tumor cells significantly *in-vitro* (**Fig.3.4 A, B**). Growth of BC-8 cells alone was slightly inhibited in the absence of 1A6 treated DCs.

The cytostatic effect of 1A6-activated PDCs was higher than SDCs upon *in-vitro* activation. Tumor growth inhibition was significantly enhanced when assay was performed with *in-vivo* primed cells; and inhibition was comparable for PDCs and SDCs when tumor
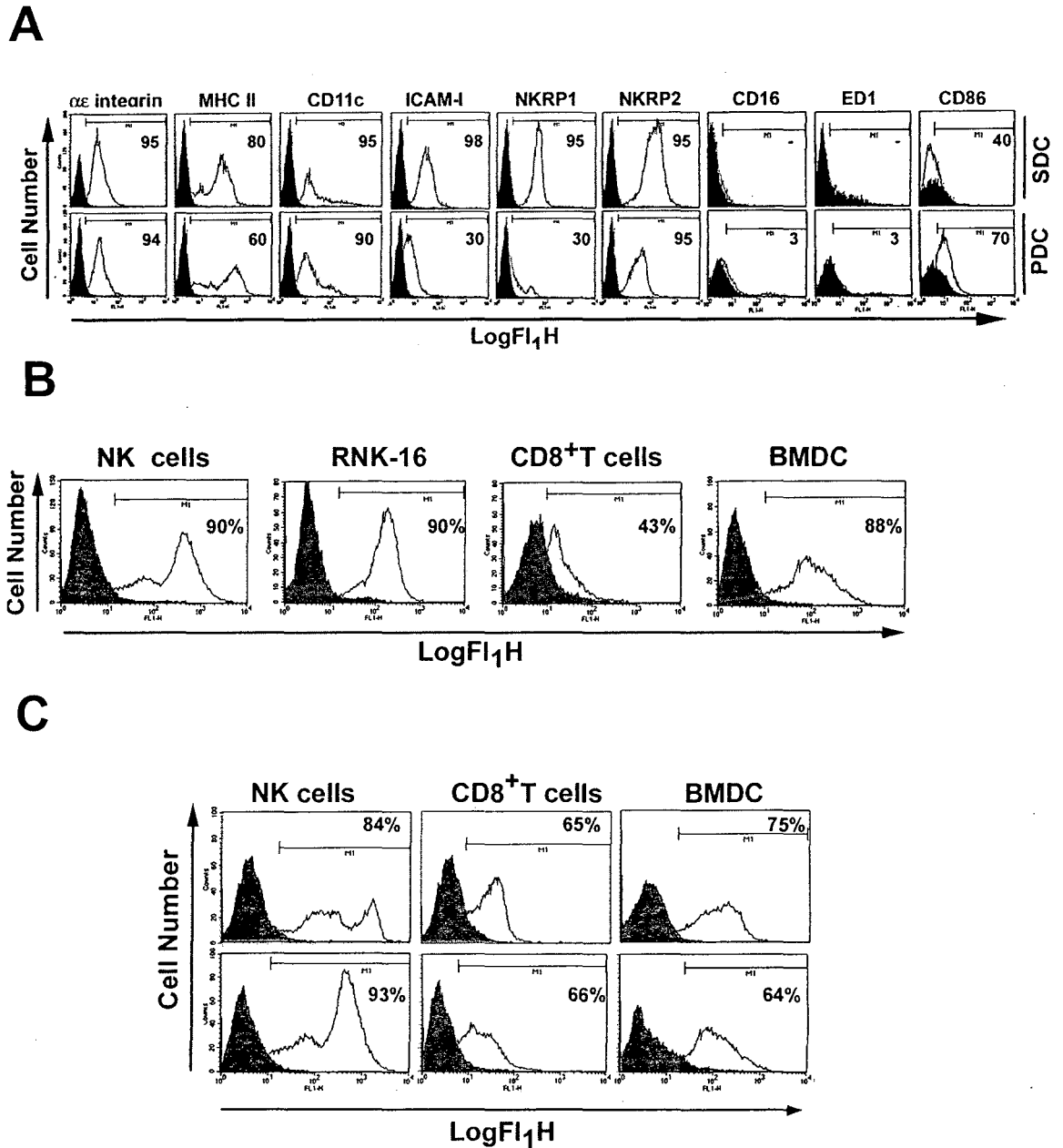
Figure: 3.1

A. Expression profile of DC markers (αε-integrin, MHC II, CD11c, ICAM-1, CD86), and expression of NKR-P1 (3.2.3Ab), NKR-P2 (mAb1A6), on freshly isolated splenic dendritic cells (SDCs) and peritoneal dendritic cells (PDCs). Macrophage marker (anti-ED1), NK cell marker (CD16) staining is also shown on pure DC population.

B. Rat NK cells, rat NK cell line (RNK-16), CD8^{+} T cells and BMDCs were stained with mAb1A6, to further establish the specificity of mAb 1A6 to NKR-P2/NKG2D.

C. Mouse NK cells, activated CD8^{+}T cells and BMDCs were stained with goat anti-mouse NKG2D polyclonal Ab (upper panel) and mAb 1A6 (lower panel). Filled histograms are secondary Ab controls, open histograms depict specific staining. Number in figures indicates % positive cells i.e. M1.

A
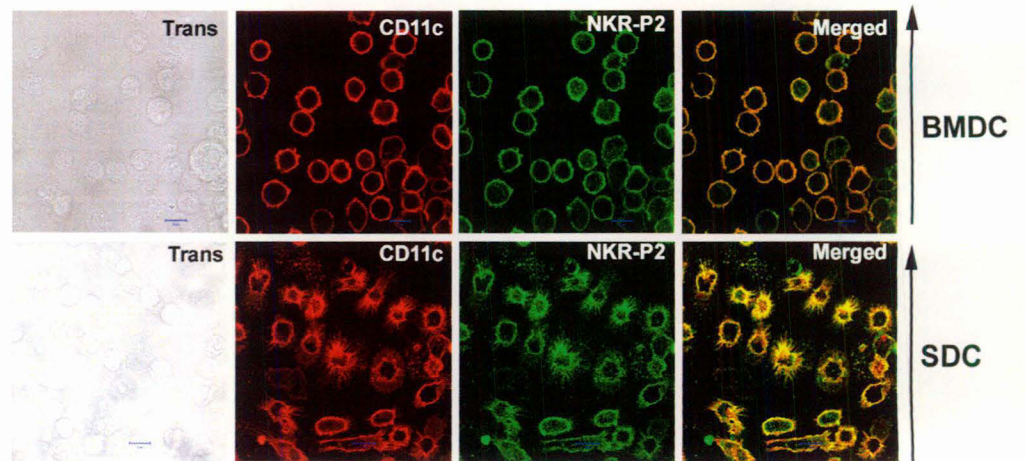
B
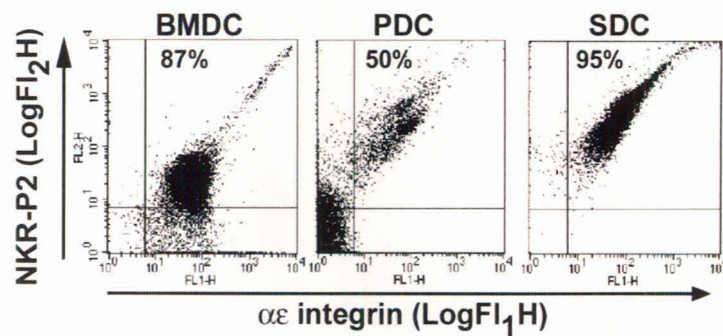
Figure: 3.2

A. Confocal scanning of rat BMDCs (upper panel) and rat SDCs (lower panel) after staining with primary anti-NKG2D polyclonal Ab (green: secondary Ab alexa-488) and counterstained with primary anti-CD11c Ab (red: secondary Ab alexa-594). Merged images are also shown that depict surface expression of NKG2D along with CD11c.

B. NKR-P2 expression on rat immature BMDCs, PDCs and SDCs. The results shown are of $\alpha\varepsilon$-integrin and NKR-P2/NKG2D positive DCs. Anti-NKR-P2 mAb was probed with alexa-594 (Fl_2H-signal) followed by staining with anti-$\alpha\varepsilon$-integrin mAb (OX-62), OX-62 Ab was probed with alexa-488 (Fl_1H-signal).
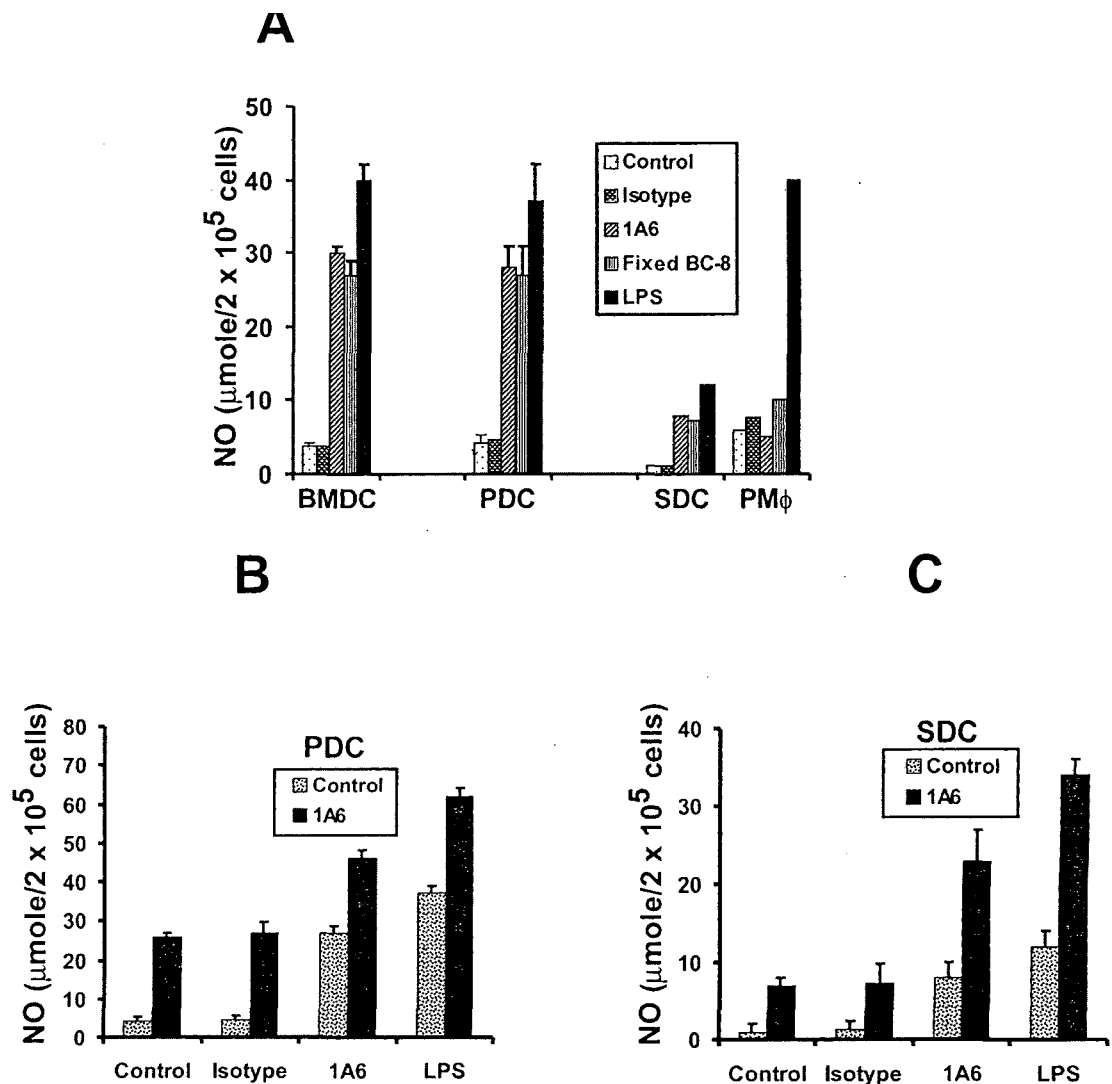
Figure: 3.3

A. Day 6 iBMDCs, Freshly isolated PDCs, SDCs and freshly isolated peritoneal macrophages were plated in 96 well plates (2×10^5 cells/well) and incubated with either isotype control IgM, anti-NKR-P2 mAb 1A6 (100 μg/ml), paraformaldehyde fixed BC-8 tumor cells (at 25:1 ratio) and LPS (1μg/ml) for 24 h. Cell free culture supernatants were used for the estimation of NO content by using Griess reagent.

B and C. Single i.p. injection of either anti-NKR-P2 mAb 1A6 or isotype control mAb (1mg of each) was given to Wistar rats (3 rats/group). After 24 h, PDCs and SDCs were harvested from experimental rats and re-stimulated with isotype Ab, anti-NKR-P2 mAb (100 μg/ml) or LPS with respect to prior stimulators. After 24h, NO content was measured in cell free culture supernatant with Griess reagent. Isotype control Ab did not show any stimulation of NO production *in-vivo*.

A

PDC

B

SDC
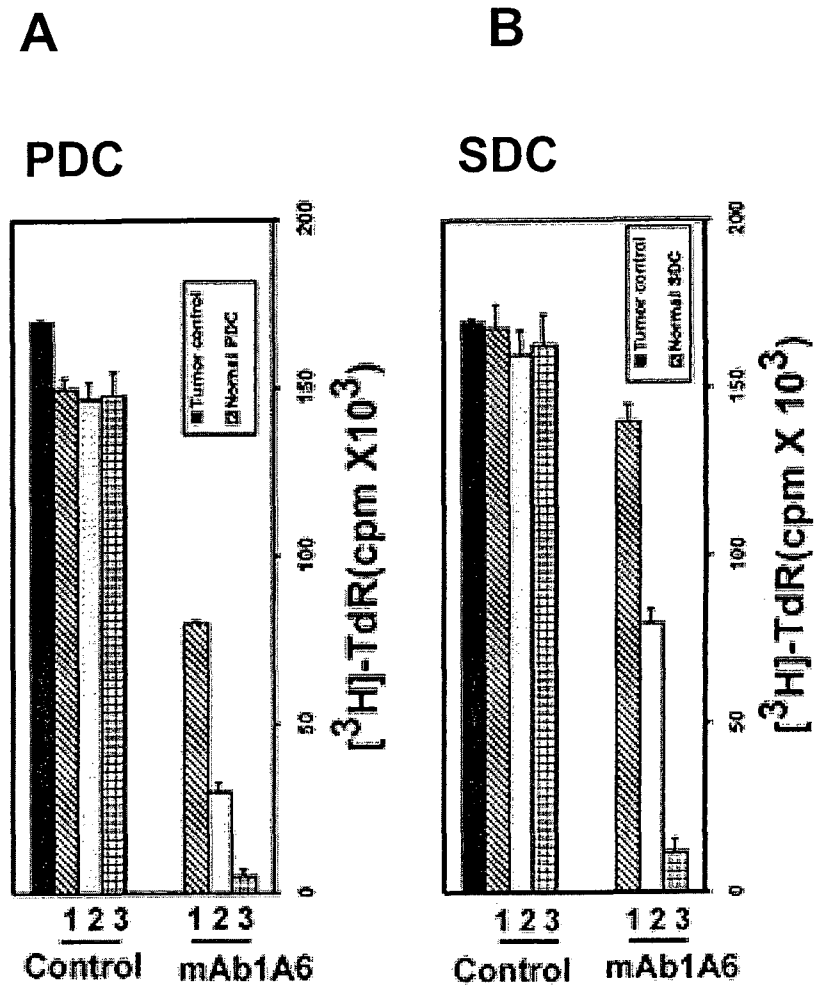
Figure 3.4

A and B: To measure the cytostatic (growth inhibitory) potential of 1A6 activated DCs against BC-8 tumor cells; PDCs and SDCs were harvested after a single i.p. injection of anti-NKR-P2 mAb (1mg/ml) or isotype control mAb injected or from normal rat (3 rats/group). Harvested PDCs and SDCs were cocultured with live BC-8 tumor cells (25-DCs:1-BC-8 cell, ratio) for 36 h.(Doubling time of BC-8 tumor cell is 16 h). [^{3}H] Thymidine was added 10 h before tumor cell harvesting. Afterward nonadherent BC-8 tumor cells were harvested and radioactivity was measured using β-counter. Decrease in the proliferation of BC-8 tumor cells was observed with 1A6 activated DCs. In figure, **1** denotes the effect of *in-vitro* 1A6 stimulated DCs; **2**, denotes the effect of *in-vivo* primed DCs; and **3** denotes the effect of *in- vivo* primed and restimulated DCs with 1A6.

inhibition was measured with restimulated primed PDCs and SDCs. The observed inhibitory effects of PDCs and SDCs from 1A6-injected rats were associated with the ability of PDCs and SDCs to produce NO *in-vitro* as described in Fig. 3.2 B,C.

3.2.3   1A6 mimics NKR-P2 ligand for DCs

To check whether mAb1A6 binds to NKR-P2/NKG2D and mimics its ligand, we performed the 1A6 binding assays upon NKG2D pre-blocking. A significant decrease in binding of 1A6 was observed with goat anti-NKG2D polyclonal Ab on SDCs **(Fig. 3.5A)**. Similarly, a significant decrease in activating potential of mAb1A6 was also observed upon blocking the NKR-P2/NKG2D with bivalent anti-NKG2D polyclonal Ab as found by decreased NO production **(Fig. 3.5B)**.

3.2.4   1A6 fails to activate NK cells and macrophages *in-vivo*

Our earlier observation (Alli et al., 2004) shows that 1A6 acts as a blocking Ab for NK cells under *in-vitro* conditions. We further checked the NK cell activation with 1A6 *in-vivo*, but could not find the upregulation of FasL and IFN-γ on splenic NK cells confirming no activation of NK with 1A6 **(Fig. 3.6A)**. Similarly, iNOS and TNF-α levels remained unchanged in splenic Mϕ upon 1A6 injection **(Fig. 3.6B)**.

3.2.5   No endotoxin contamination in 1A6 mAb

To rule out the possibility of endotoxin contamination in mAb1A6 preparation, we performed LAL assays and the endotoxin contamination was always below the permissible limits (less than 0.125EU). Furthermore, boiling of 1A6 (100°C, 20 min) completely abrogates its activity whereas retains its potential upon polymyxin-B (50 μg/ml) treatment. In parallel assays LPS activity was significantly abrogated with polymyxin-B and remained unaltered upon boiling (100°C, 20 min) **(Fig. 3.7)**.

3.2.6   1A6 dependent tumor killing is mediated by Nitric Oxide

To illustrate mAb1A6 mediated DCs activation and tumor cell killing, we have studied activation of immature BMDCs with 1A6. **Figure 3.8 A**, demonstrates upregulation of iNOS transcript; and **Figure 3.8 B** show the iNOS protein upregulation upon 1A6 cross-linking on immature BMDCs. Induction of iNOS transcript and protein was not observed with Isotype control Ab at similar concentrations. However, comparable iNOS induction was detected after coculture with fixed BC-8 cells and LPS, which were used as positive controls.

A

LogFl₁ NKRP2

B

| mAb1A6
(100μg/ml) | - | + | + | - |
| anti-NKG2D
(100 μg/ml) | - | - | + | + |
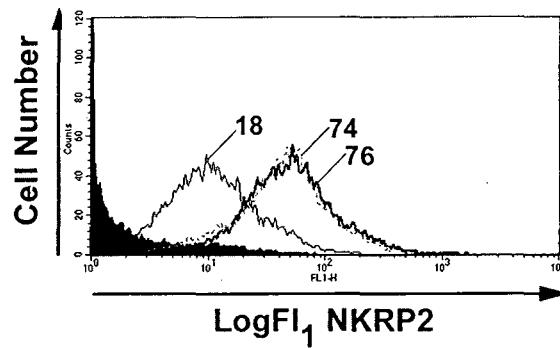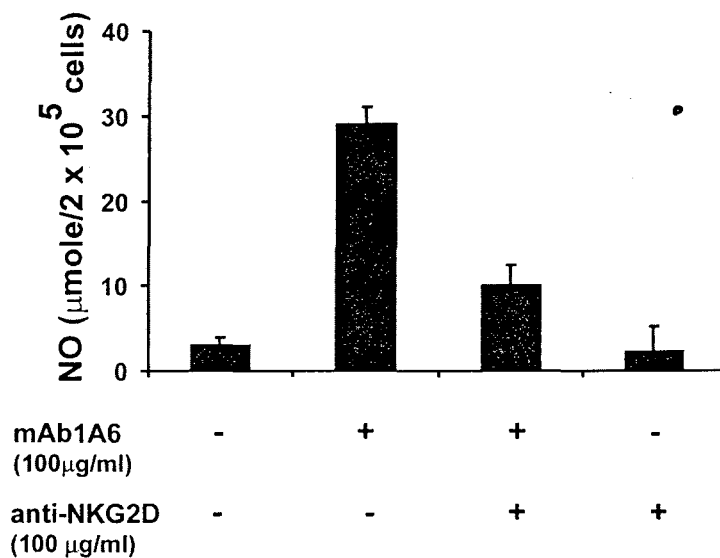
Figure: 3.5

A. Inhibition of 1A6 binding upon NKG2D pre-blocking on DCs: Formaldehyde fixed SDCs were pre-blocked with goat anti-mouse polyclonal anti-NKG2D Ab (Bivalent IgG) for 2 h, followed by staining with mouse mAb1A6 (Pentavalent IgM). (Filled histogram denotes anti-mouse secondary Ab control, bold line – binding of 1A6, thin line – binding of 1A6 on SDCs pre-blocked with anti-NKG2D bivalent Ab, broken thin line --- binding of 1A6 on SDC pre-blocked with mouse IgG). Binding of IgM-mAb 1A6 was probed with secondary Ab alexa-488. Numbers in figure indicate the respective MFI for each histogram as analysed by FACS.

B. Inhibition of 1A6 activity (decreased NO production) upon pre-blocking of NKG2D/NKR-P2 with polyclonal bivalent Ab: Day 6 attached BMDCs were pre-blocked in incomplete media for 2 h with goat anti-mouse NKG2D polyclonal Ab before stimulation with IgM-mAb 1A6 as indicated in the figure. After 24 h of stimulation with mAb1A6, NO content was measured in cell free culture supernatant with Griess reagent.

A

Control   1A6

FasL

IFN-γ

LogFl₁

B

Control   Isotype   1A6

iNOS

TNF-α

LogFl₁H
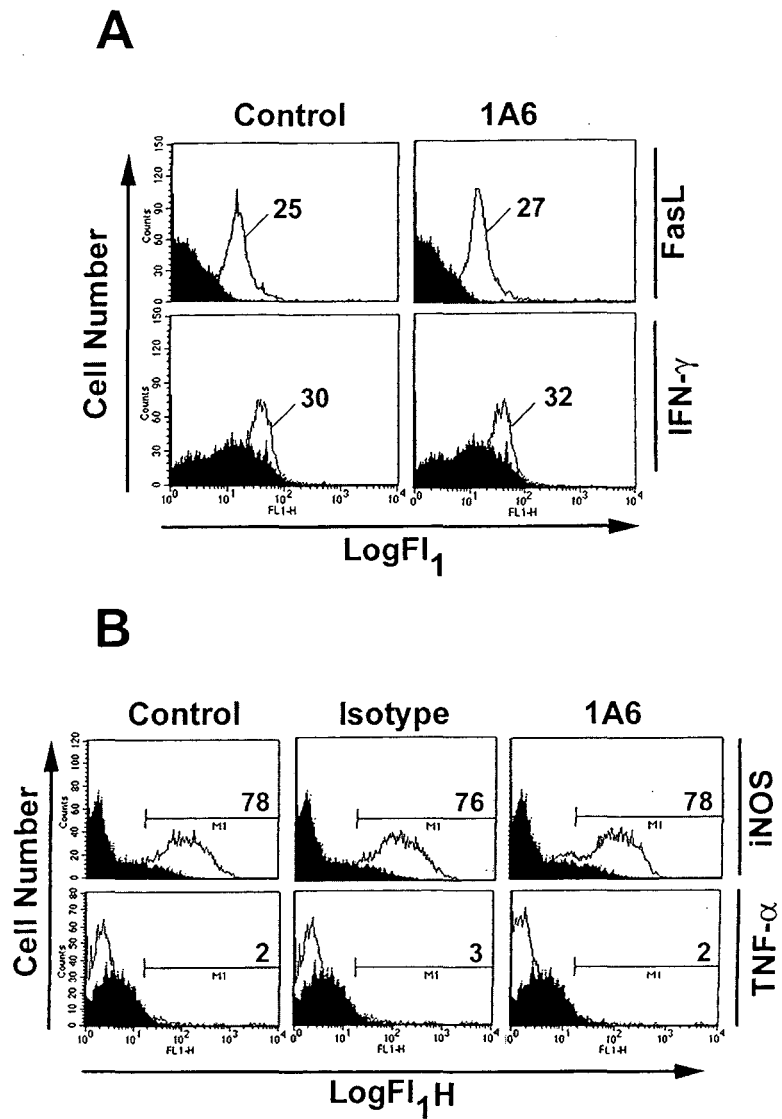
Figure 3.6

To demonstrate the non-agonistic nature of 1A6 on NK cells and macrophages, the *in-vivo* analysis of splenic NK and splenic macrophage activation markers was examined upon 1A6 injection in wistar rats.

A. Staining of NK activation surface molecules FasL (CD95L), and the staining of intracellular IFN-γ for splenic NK cells upon 1A6 injection.

B. Staining of TNF-α and iNOS for splenic macrophages upon 1A6 injections. (Filled histograms represent secondary Ab controls, open histogram show the specific staining with respective Ab as marked in figure. Numbers in figure represent the MFI of each histogram under marker M1). (1mg/ml/rat/day, 5-injections each in 3 rats were given along with appropriate controls). Appropriate secondary Ab (alexa-488) was used to probe the specific staining.

NO (μmole/2 x 10^5 cells)

50
40
30
20
10
0

1A6   LPS

+ Boiling   -   -   +   -   -   +   -   -
+ Polymyxin B   -   -   -   +   -   -   +   +
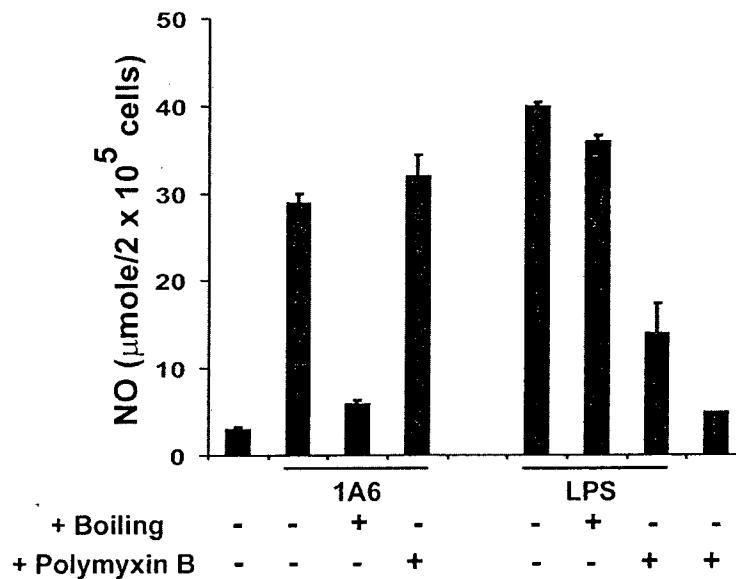
Figure 3.7

Anti-NKR-P2 mAb1A6 preparation is free of endotoxin contamination.
BMDCs produce NO upon stimulation with 1A6. BMDCs were stimulated with 1A6 and LPS. 1A6 in presence of 50 μg/ml peptide-polymyxin B (PMB) and heat-denatured 1A6 form (100°C, 20 min) were used as BMDC stimulators. Upon boiling agonistic nature of IgM-1A6 was completely abrogated due to its proteinaceous nature, whereas polymyxin B failed to neutralize its agonistic nature. In the parallel assay heat inactivation did not inhibit LPS activity significantly, which was significantly brought down with PMB-neutralized LPS. Representative bar diagram is indicative of several experiments that were carried out to ensure no endotoxin contamination in 1A6, which was purified with ammonium sulphate precipitation and protein A sepaharose affinity column throughout the study.

A

	Control	Isotype	1A6	BC-8	LPS
GAPDH						~200 bp
iNOS					

B

iNOS	~135 kDa

β-Actin
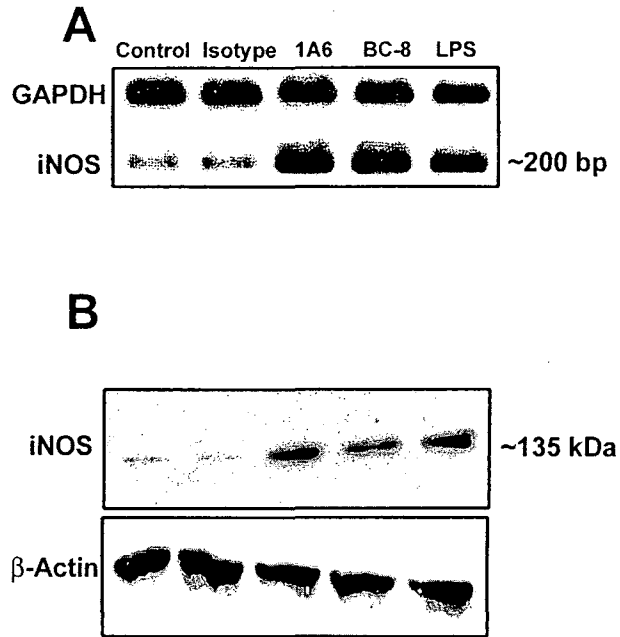
Figure: 3.8

A and B. Upregulation of *iNOS* transcript and protein upon NKR-P2 cross-linking with 1A6. Day6 attached BMDCs were incubated with isotype control mAb or with 1A6 for 24 h. Total RNA and proteins were isolated from similar experimental set up and subjected to RT-PCR (using iNOS specific primers) and immunoblotting (using rabbit anti-mouse iNOS Ab). GAPDH and β-Actin detection were used to ascertain equal amount of cDNA template and protein in RT-PCR and immunoblotting. Fixed BC-8 cells (at 25:1 ratio) in coculture with attached BMDCs and LPS (1μg/ml) were used as positive controls for iNOS induction in attached BMDCs.

As a result of iNOS induction, 6~7 fold increase in NO content was observed in 1A6 treated BMDCs. NO secretion was significantly abrogated upon pretreatment of BMDCs with irreversible iNOS inhibitor W1400. Similarly, NO levels in BC-8 and LPS treated BMDCs were also inhibited with W1400 (Fig. 3.9A). Furthermore, we also determined the cytostatic activity of 1A6-activated BMDCs. Figure 3.9 B demonstrates cytostatic activity of BMDCs upon 1A6 cross-linking. Tumor cell proliferation was significantly inhibited upon NKR-P2 cross-linking, which was abrogated upon W1400 treatment. However Isotype control treated and untreated BMDCs inhibited slightly the growth of tumor cells.

Since NO is a well known mediator of apoptosis in tumor cells, we assayed apoptosis in tumor cells cocultured with 1A6 activated BMDC by flow cytometry after PI staining and also by Annexin V staining. Interestingly, enhanced apoptosis in tumor cells was observed with mAb1A6 treated BMDCs. Apoptosis was significantly abrogated upon pretreatment of BMDCs with W1400 before 1A6 stimulation. The presented data clearly elucidate NO as the potent mediator of DCs induced apoptosis in tumor cells (Fig.3.10).

3.2.7 NKR-P2 cross-linking activates p38K, AKT, ERK and translocation of NF-κB

To determine the signaling pathways involved in the production of NO upon 1A6 cross-linking in BMDCs, we measured NO content in PD98059, Ly294002 and SB203580 treated BMDCs along with experimental controls and found a significant reduction in NO production with these inhibitors, which confirms the involvement of MAP kinase in 1A6 mediated BMDCs activation. Significant inhibition of NO production was also observed with Ca^{2+} chelator EGTA, protein kinase C inhibitor, H7 and with protein tyrosine kinase inhibitor, Genistein (Fig. 3.11A).

We further studied the effect of PD98059, Ly294002 and SB203580 on iNOS levels. Significant inhibition of iNOS expression was observed upon pretreatment of BMDCs with ERK1/2 inhibitor PD98059, AKT kinase inhibitor Ly294002 and only a slight downregulation of iNOS was observed with p38 kinase inhibitor SB203580 (Fig. 3.11B).

We have further examined phosphorylation kinetics of p38, AKT and ERK1/2 upon 1A6 cross-linking and compared it with LPS mediated signal transduction at similar time points by immunoblotting. Upon 1A6 stimulation, maximum pP38 was found at 45-60 min, whereas LPS caused significant phosphorylation at 10 min. Both 1A6 and LPS mediated p38 phosphorylation was inhibited by imidazole SB203580. However, complete inhibition was

A **B**
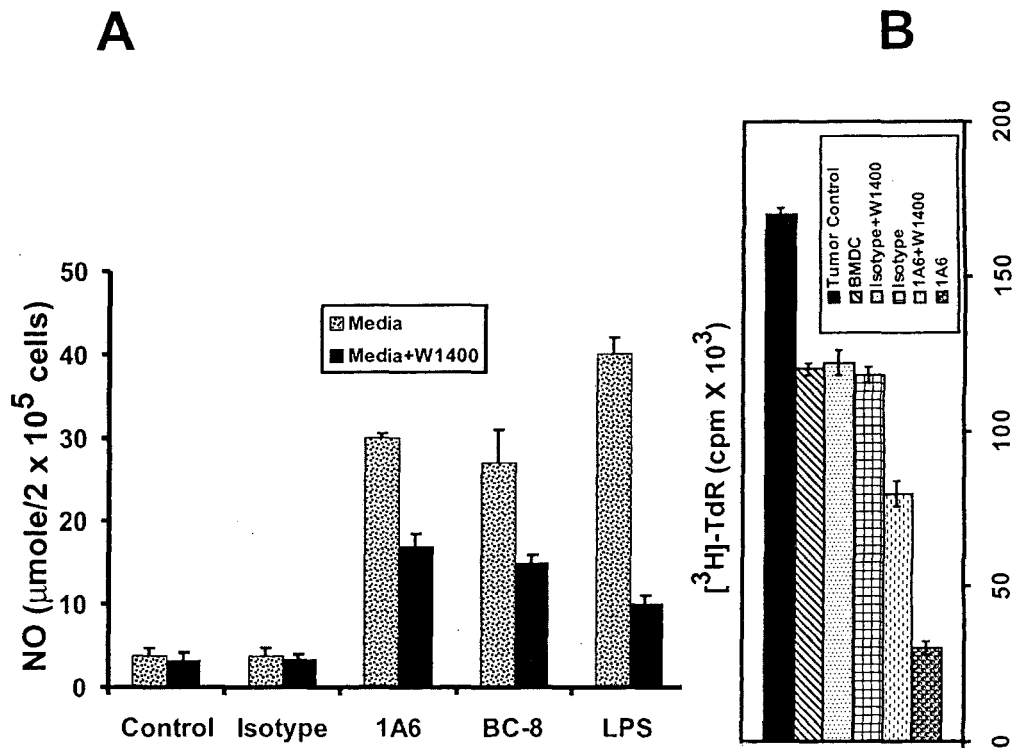
Figure: 3.9

A. NO secretion upon NKR-P2 cross-linking was inhibited with W1400. Day6 attached BMDCs were pretreated with W1400 (5μM, 45min) afterward stimulated with mAb 1A6. Fixed BC-8 cells (at 25:1 ratio) and LPS in the absence or presence of W1400 were also used as positive control. Cell free culture supernatants were collected from triplicate experimental setup after 24 h and NO content was measured by Griess reagent.

B. Cross-linking of NKR-P2 on BMDCs mediate cytostatic action through NO. BMDCs were pretreated with W1400 (5μM, 45min) and stimulated with mAb 1A6 for 6 h along with experimental controls as indicated in the figure. Subsequently, BMDCs were co-incubated with live BC-8 tumor cells (doubling time 16 h) for 36 h. [^{3}H] Thymidine was added 10 h before tumor cell harvesting.

Control	BMDC	Isotype	1A6	1A6+W1400	W1400
3%	16%	16%	50%	30%	5%

DNA content (Fl$_2$A)

3%	15%	15%	85%	25%	15%

Annexin V (LogFl$_1$H)
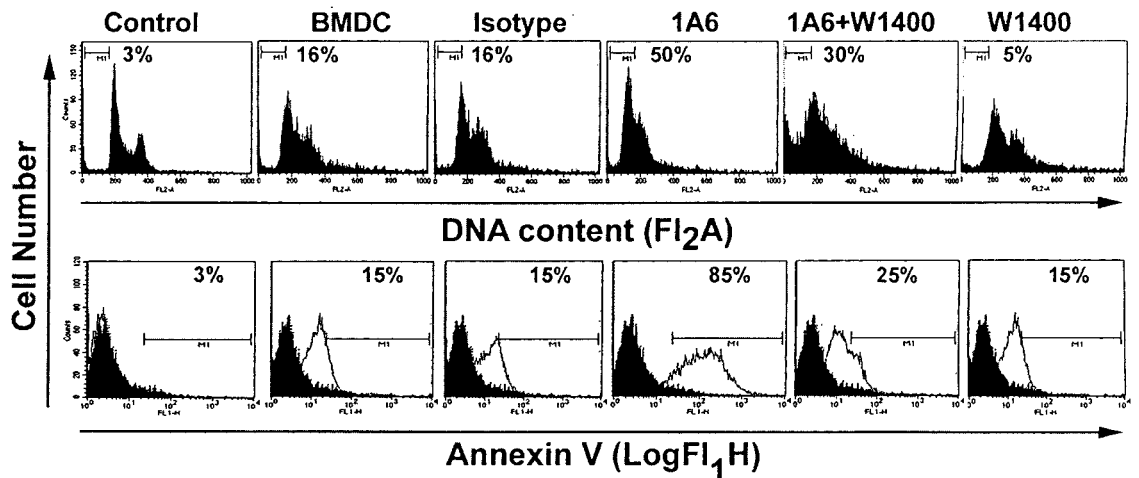
Figure: 3.10

1A6 activated BMDCs trigger tumor cell apoptosis through NO. Attached BMDCs were pretreated with W1400 (5μM) for 45min (irreversible iNOS inhibitor) and stimulated with mAb1A6 for 6 h along with experimental controls as indicated in the figure. Subsequently, firmly attached BMDCs were co-cultured with non-adherent BC-8 tumor targets at 25:1 ratio for 24 h and 18 h to estimate hypoploidy and phosphatidylserine externalization with propidium iodide and Annexin-V staining separately. For DNA analysis BC-8 tumor cells were fixed and permeabilized in 80% methanol whereas Annexin-V staining was performed on live tumor cells. (Phosphatidylserine externalization is an early event of apoptosis and DNA degradation is a late event in apoptosis, however both the assays are the hallmark of cellular apoptosis). Upper Panel shows DNA degradation and the % apoptotic tumor cells under the marking M1. Lower panel denotes % Annexin-V positive live BC-8 tumor cells in M1 marked open histograms. Filled histograms in the lower panel show autofluorescence of BC-8 tumor cells.

A

B
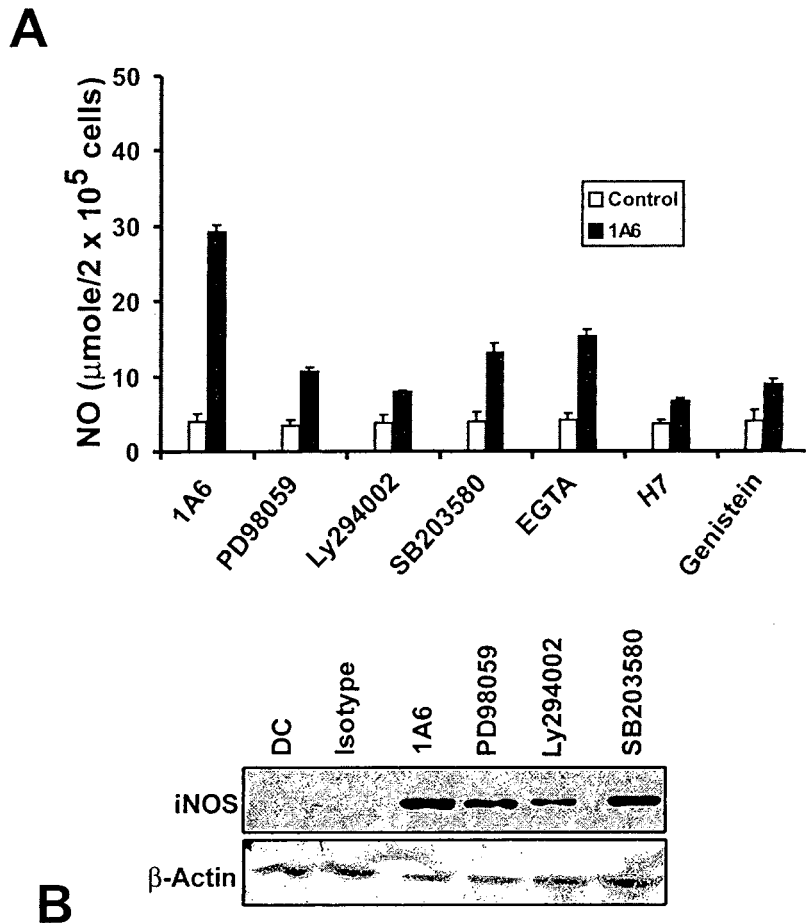
Figure: 3.11

A. Effect of pharmacological inhibitors PD98059, Ly294002, SB203580, EGTA, H7 and Genistein on 1A6 induced NO production by BMDCs. Attached day 6 BMDCs were treated with PD98059 (20 μM), LY294002 (50 μM), SB203580 (10 μM), EGTA (2mM), H7 (10 μM), Genistein (10μM) or medium alone for 1 h before stimulation with mAb 1A6 (100 μg/ml). After further 24 h incubation, cell free culture supernatants were collected and NO content was measured by Griess reagent.

B. Effect of PD98059, LY294002 and SB203580 on mAb 1A6 induced iNOS expression. BMDCs were pretreated with or without PD98059 (20 μM), SB203580 (10 μM) or LY294002 (50 μM) for 1 h before stimulation with mAb1A6 for 12 h. Cell lysates were analyzed by immunoblotting using rabbit anti-mouse iNOS Ab.

observed in the case of 1A6 at 60 min. Chomone Ly294002 is a specific inhibitor of PI3 kinase and blocks activation of AKT kinase. pAKT form was significantly upregulated at 45 min upon 1A6 cross-linking as well as by LPS treatment. Ly294002 completely abrogated the 1A6 mediated AKT phosphorylation at 60 min and a significant blocking was also observed in LPS mediated activation (**Fig. 3.12**). .

The ERK pathway is known to be important in many cell types as regulator of cell survival. ERK1/2 phosphorylation was detected within 10 min after 1A6 or LPS stimulation. LPS induced stronger activation of ERK2 at 60 min and 1A6 induced ERK1 activation strongly till 45 min. Strong activation of both ERK1 and ERK2 was observed at 45 and 60 min with LPS, whereas significant ERK2 activation was observed only at 60 min with 1A6. However, flavanoid PD98059 inhibited significantly both ERK1/2 phosphorylations at 60 min (**Fig. 3.12**).

NK-κB plays a significant role in DCs activation; therefore we have investigated the role of NF-κB in mAb 1A6 induced BMDCs activation. The transcription factor NF-κB is bound to IκB-α in the cytoplasm and retained there in an inactive form. LPS stimulation results in degradation of IκB-α and as a result free NF-κB translocates to the nucleus and activates transcription of various genes. IκB-α degradation was observed within 30 min upon LPS treatment. However, significant degradation of IκB-α was only observed at 60 min upon 1A6 cross-linking indicating similar but slow activation of NF-κB with 1A6 in comparison to LPS (**Fig. 3.13**).

To confirm NF-κB translocation, nuclear extracts of stimulated BMDCs were immunoblotted with NF-κB p65. Upon LPS stimulation NF-κB was detected at 30 min, whereas in 1A6 stimulated cells, the band was detected at 60 min (**Fig. 3.13**). Hence, IκB-α degradation correlates very well with NF-κB translocation and thus confirms the activational translocation.

3.2.8   1A6 induces maturation of DCs and proliferation of autologous T cells

BMDCs and SDCs without any stimulus or with isotype control mAb showed the typical phenotypes of immature BMDCs and SDCs. However, upon NKR-P2 cross-linking with mAb1A6, both BMDCs and SDCs undergo maturation in 24 h. MHC II, B7-2 and CD1a specific mAbs detected enhanced surface expression of these maturation markers on 1A6 treated DCs. Average mean percentage of MHC II$^+$ cells were 20% and 30% for BMDCs and SDCs, while 1A6 cross-linking increased this to 92% and 97% respectively, in 24 h.

64

Time (min)	0	10	30	45	60	+SB203580	
1A6							pp38
LPS							pp38
Total							p38

+Ly294002

1A6							pAKT
LPS							pAKT
Total							AKT

+PD98059

1A6							ppERK1/2
LPS							ppERK1/2
Total							ERK1/2
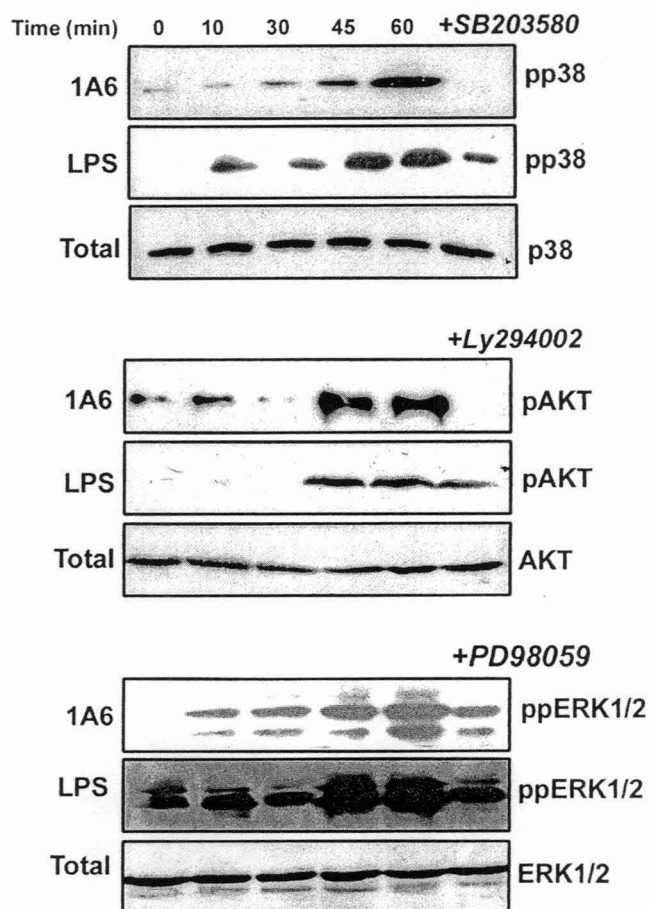
Figure: 3.12

1A6 induces phosphorylation of p38K, AKT and ERK1/2. BMDCs were treated with 1A6 (100 μg/ml) or LPS (1 μg/ml) separately and subjected to immunoblotting with specific Abs (control and phospho-form specific Ab). As indicated in the Figure, attached BMDCs were pretreated with SB203580 (upper panel), Ly294002 (middle panel), and PD98059 (lower panel) for 45 min before exposure to 1A6 or LPS. The panels show detection of phosphorylated form of pp38, pAKT and pERK1/2 along with unphosphorylated forms p38, AKT and ERK1/2 as controls at different time intervals (0, 10, 30, 45, 60 min) along with inhibitors. Inhibitor treated BMDCs were loaded in right most well as indicated in figure. For the detection of total form and phosphorylated form of kinases the protein samples were loaded in parallel wells in equal amount.
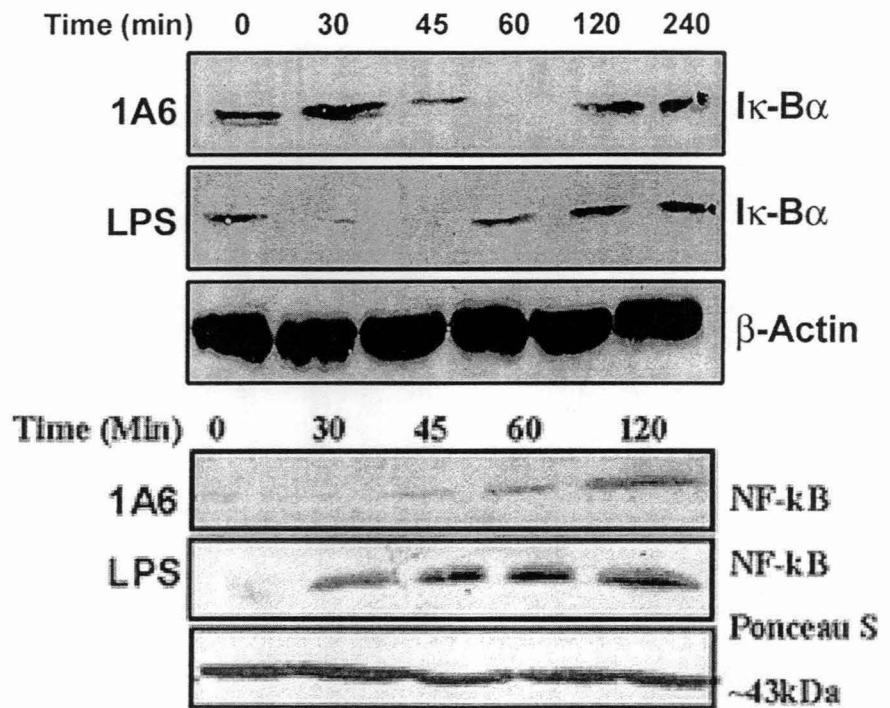
Figure: 3.13

Role of NF-κB in 1A6 induced BMDC activation. 1A6 treatment induces the degradation of Iκ-Bα in immature BMDCs. Attached BMDCs were treated with 1A6 (100μg/ml) and LPS (1μg/ml) for different time points (0,30,45,60,120,240 min) as indicated in figure, total cellular protein was run on SDS-PAGE followed by protein transfer to membrane. Iκ-Bα signal was detected with specific mAb, along with detection of β-actin, as equal loading control for total protein. From the similar experimental setup nuclear extracts from attached BMDCs were prepared and subjected to immunoblotting with rabbit anti-NF-κBp65 polyclonal Ab (lower panel). Ponceau *S* blot of the nuclear extract from 1A6 treated BMDC is shown as the equal loading control.

Similarly B7.2 levels were increased from 22% to 96% for BMDCs and 44% to 97% for SDCs. Upon 1A6 cross-linking CD1a expression was also enhanced from 5% to 22% for BMDCs and 11% to 25% for SDCs. Coculture of adherent BMDCs and SDCs with fixed BC-8 tumor cells at 1:25 ratio also induced upregulation of MHC II, B7-2 and CD1a and was used as positive control to assess maturation **(Fig. 3.14)**.

After evaluating the phenotypic maturation of BMDCs and SDCs, we tested the ability of BMDCs and SDCs to stimulate T cell proliferation in an autologous MLR assay. BMDCs and SDCs with isotype mAb exhibited a higher T cell proliferation activity. Upon 1A6 cross-linking ~3 fold proliferation was detected with BMDCs, whereas ~ 4 fold proliferation was recorded with SDCs. Stimulatory capacity of 1A6 was significantly inhibited upon 1A6 neutralization with recombinant rNKR-P2 protein for both BMDCs and SDCs. rNKR-P2 protein did not induce stimulation of BMDCs **(Fig. 3.15A)**. The T-cell stimulatory capacity of SDCs was found to be higher than BMDCs. To exclude the possibility of a direct action of 1A6 on T cells, autologous T cells treated with 1A6 were also used as responder in MLR assay. Cellular aggregation in response to surface Ags signaling is a synchronized process and essential for cell-cell interaction and adhesion. Upon treatment of 1A6 both BMDCs and SDCs undergo homotypic clustering. BMDCs cultures led to formation of large homotypic clusters, whereas SDCs showed comparatively smaller homotypic clusters in 6 h. This phenomenon was not observed with Isotyope-matched mAb **(Fig. 3.15B)**.

3.2.9 Role of p38 MAPK, AKT and ERK pathways in 1A6 mediated DCs maturation

To investigate the role of MAPK signaling pathway in 1A6 induced BMDCs maturation, we pretreated BMDCs with PD98059, Ly294002 and SB203580 and evaluated expression profile of MHC II and B7-2 upon 1A6 treatment using double staining. A 23% reduction in MHC II/ B7-2 expression was found with ERK1/2 inhibitor on BMDCs. Ly294002 inhibited double staining to 37% on BMDCs. SB203580 inhibited expression to 35% on BMDCs **(Fig. 3.16A)**. Taken together, these results suggest involvement of MAP kinase signaling in 1A6 mediated DCs maturation.

3.2.10 1A6 cross-linking reduces endocytic capacity of DCs

iDCs possess high endocytic capacity whereas upon maturation, the endocytic function is reduced and the Ags presentation function increases. We have evaluated the effect of 1A6 treatment on endocytosis by monitoring uptake of Zymosan-FITC in SDCs. At 4°C the endocytic capacity is inhibited, however, at 37°C both untreated and isotype control
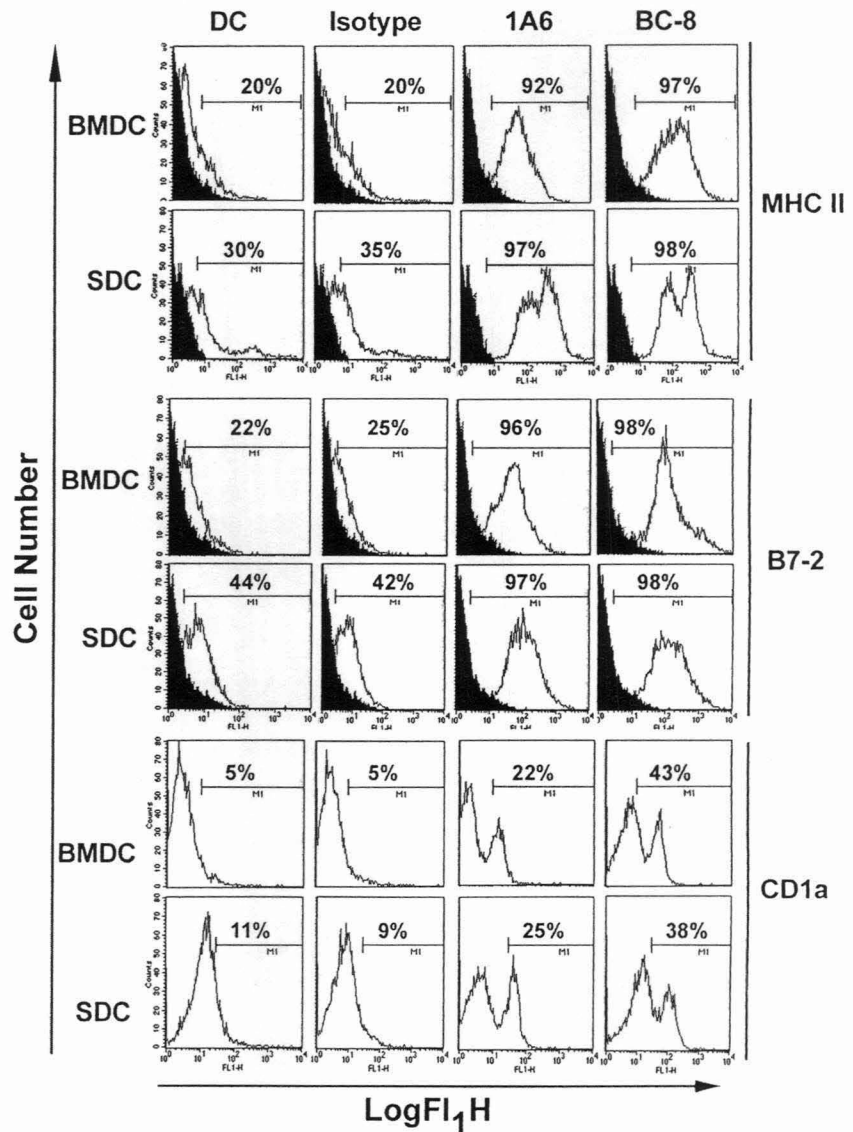
Figure: 3.14

Induction of DC maturation markers.

Attached BMDCs and SDCs were incubated with medium alone, Isotype control mAb, 1A6 (100µg/ml) and with fixed BC-8 cells at 25:1 ratio (BC-8: DC) for 24 h. Treated BMDCs and SDCs along with control were fixed in 1% formaldehyde, washed and probed with MHC II, B7-2, and FITC-tagged CD1a Ab. Cells were further probed with appropriate secondary Ab (Alexa-488). Marker M1 (open histogram) represents the percentage positive cells, (filled histogram denotes the secondary Ab control that were used to probe the detection of MHC II and B7-2 primary Ab).
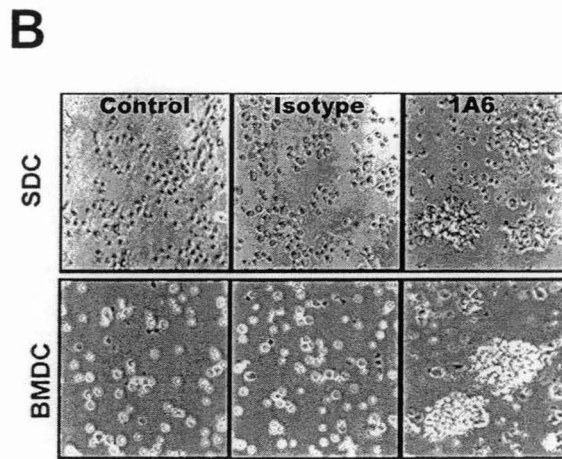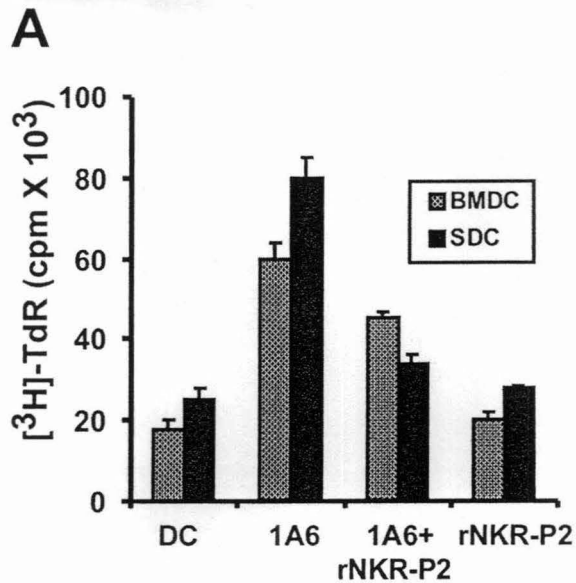
Figure: 3.15

A. 1A6 enhances the capacity of DCs to activate T cells. BMDCs and SDCs were treated with 50 μg/ml of 1A6; 50 μg/ml 1A6 + 50 μg/ml rNKR-P2 and 50 μg/ml rNKR-P2 protein alone for 24 h. Activated DCs along with experimental controls were incubated with autologous CD3^{+} splenic T cells at 1:20 (DC: T) ratio. [^{3}H] Thymidine was added 12 h before harvesting of T cells that were incubated for 36 h with activated DCs. After T cells harvesting on silicone membrane the proliferation index was counted in β-counter with enhancer-cocktail. Results are shown as mean cpm x 10^{3} ± SD, and are representative of 3 similar experiments.

B. Anti-NKR-P2 mAb 1A6 enhances homotypic clustering in DCs. Non-adherent BMDCs and SDCs were incubated with isotype control mAb or with 1A6 or left untreated for 6 h and then photographed at 40X in 6-well culture plates after mild-shaking. As observed in photograph,1A6 treatment induces homotypic (association of similar cell type) clustering in nonadharent DCs.

A

MHC II (LogFl$_2$H)

Control Isotype 1A6 +PD98059 +Ly294002 +SB203580

3% 3% 98% 75% 62% 65%

B7-2 (LogFl$_1$H)

B

Cell Number

DC Isotype 1A6 BC-8 LPS

54% 55% 14% 15% 14%

M1 M1 M1 M1 M1

LogFl$_1$H
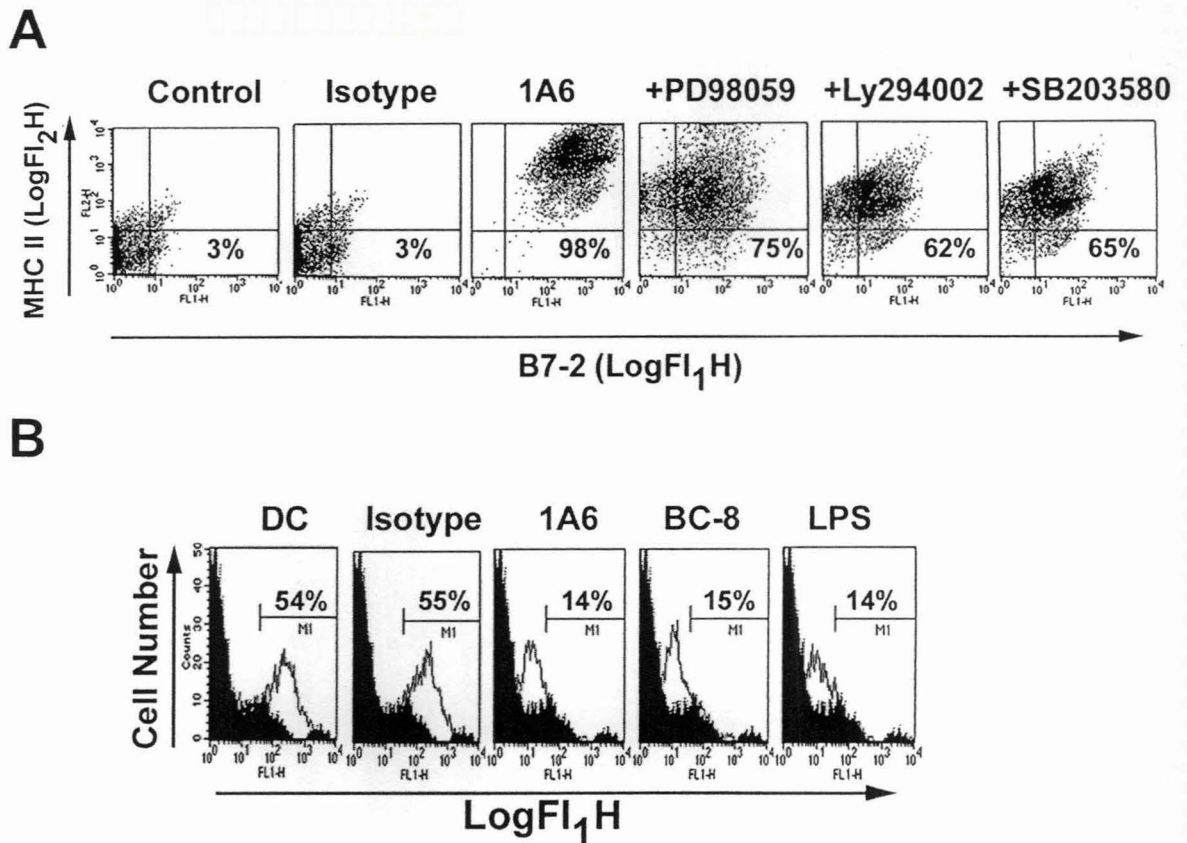
Figure: 3.16

A. Effect of PD98059, LY294002 and SB203580 on 1A6 induced upregulation of maturation markers on BMDCs. Immature day6 BMDC were pre-treated with the inhibitors (PD98059, LY294002 and SB203580) for 45 min, and stimulated with isotype control mAb and mAb 1A6 for 24 h. DCs were fixed in 1% formaldehyde and probed with mouse anti-MHC II mAb and rat anti-B7-2 mAbs simultaneously. Anti-mouse alexa-594 was used for detection of MHC II signal (Fl$_2$H) and anti-rat alexa-488 was used for the detection of B7-2 (Fl$_1$H) by FACS. Double staining in the figure shows the surface expression of MHC II and B7-2 and its inhibition with respective inhibitors. Numbers in the figure denote the % positive population in the upper right quadrant.

B. Downregulation of endocytosis by DCs upon NKR-P2 cross-linking with 1A6. Attached SDCs were incubated with isotype control mAb, 1A6, with fixed BC-8 cells (25:1 ratio) and LPS for 24 h. DCs were harvested, washed with PBS and analyzed for Zymosan-FITC uptake. Harvested DCs were incubated with Zymosan-FITC (1 mg/ml) at 37°C (in CO$_2$ incubator) or at 4°C (in ice) for 60 min in incomplete media, in 1.5ml eppendorf tubes. After washing with chilled PBS 5-times, cells were analyzed by FACS to measure specific uptake of Zymosan-FITC and non-specific binding of Zymosan-FITC to DCs. Filled histogram and open histogram represent non-specific binding at 4°C and specific uptake at 37°C respectively. Numbers in the figure denote the % positive population under the marking M1 that represents the specific uptake of fluorescent-zymosan by DCs, as calculated in overlay histogram (open histogram as denominator) by cell quest software.

treated SDCs showed significant uptake, whereas cross-linking of NKR-P2 with 1A6 inhibited the Zymosan-FITC uptake. Similar inhibition of endocytosis was observed upon coculture of SDCs with fixed BC8 tumor cells. LPS matured SDCs were used as positive control in the assay **(Fig. 3.16B).**

3.2.11 1A6 triggers synthesis of inflammatory cytokine by DCs

DCs maturation stimuli are known to induce the secretion of inflammatory cytokines, such as IL-12, TNF-α, IFN-γ and IL-1β. Low concentration of cytokines was detected in the culture supernatants of BMDCs and SDCs. Upon 1A6 stimulation *in-vitro* significantly higher amounts of IL-12, IFN-γ and IL-1β were produced by BMDCs, whereas SDCs produced IL-12, IFN-γ, IL-1β and TNF-α also. Isotype matched control mAb failed to stimulate BMDCs and SDCs under similar conditions. Upon coculture with fixed BC-8 cells significantly higher levels of these cytokines were produced by both BMDCs and SDCs (Table 3.1). Cytokine elevation was also confirmed by semiquantitative RT-PCR for both BMDCs and SDCs **(Fig. 3.17).**

3.2.12 Immunotherapeutic potential of 1A6 in anti-tumor responses

To investigate functional status of DCs *in-vivo* 1A6 was injected in normal rats. 1A6 injected OX62 positive SDCs were found to possess enhanced expression of iNOS and B7-2 **(Fig. 3.18A),** which demonstrates higher activation status of DCs *in-vivo* and therefore could be responsible for faster regression of transplantable tumor.

To evaluate the therapeutic potential of agonistic 1A6, we examined its anti-tumor activity in AK-5 tumor regression model. Rats were implanted with 3×10^6 tumor cells at s.c. site and tumor growth was monitored till its complete regression. Interestingly co-administration of 1A6 either by s.c. or i.p. along with 3×10^6 tumor cells (s.c.) resulted in slow growth and faster regression of the tumor. 1A6 mediated rapid regression of tumor was also found to be associated with the appearance of early necrotic spots on solid tumors **(Fig. 3.18B).**

AK-5 is highly virulent upon i.p. transplantation and leads to 100% mortality of rats (Khar et al., 1998). 1A6 co-administration (i.p./s.c.) cured 60% of animals and showed delayed death in 40% rats, whereas all control animals died by early ascites development **(Fig3.19A).** Similarly ZAH hepatoma being less virulent, hence higher number (2×10^7) of cells was transplanted to develop ascites in rats. A prolonged survival of the hosts was seen upon 1A6 co-administration whereas all control animals died by day10 **(Fig3.19B).**

		Cytokine (pg/ml/10^6 cells)			
		IL-12	**TNF-α**	**IFN-γ**	**IL-1β**
BMDC	**Control**	3.5 ± 1	17 ± 2	7 ± 3	15 ± 7
	Isotype	4.2 ± 2.0	20 ± 1	9 ± 2	18 ± 4
	mAb1A6	77 ± 3	23 ± 4	80 ± 3	121 ± 3
	BC-8	102 ± 8	82 ± 5	27 ± 7	80 ± 4
SDC	**Control**	7.3 ± 5	30 ± 2	18 ± 4	16 ± 3
	Isotype	10.3 ± 2	32 ± 1	21 ± 7	16 ± 7
	mAb 1A6	54 ± 3	92 ± 18	121 ± 5	223 ± 16
	BC-8	87 ± 1	120 ± 107	316 ± 8	240 ± 7

Table 3.1.

Release of various cytokines by DCs upon stimulation with mAb1A6. Attached BMDCs or SDCs were incubated with 100 μg ml^{-1} isotype control Ab, or 1A6 for 24 h. Control DCs were left untreated or co-cultured with fixed BC-8 cells (25:1 ratio) for 24 h and cytokine levels were measured in cell-free supernatant by enzyme-linked immunofiltration assay. Data are expressed as pg ml^{-1} 10^{-6} cells ± standard deviation. $P < 0.05$, student's t-test. Similar results were obtained in three independent experiments.

	BMDC			SDC		
	Control	1A6	BC-8	Control	1A6	BC-8
IL-12						
TNF-α						
IFN-γ						
IL-1β						
GAPDH						
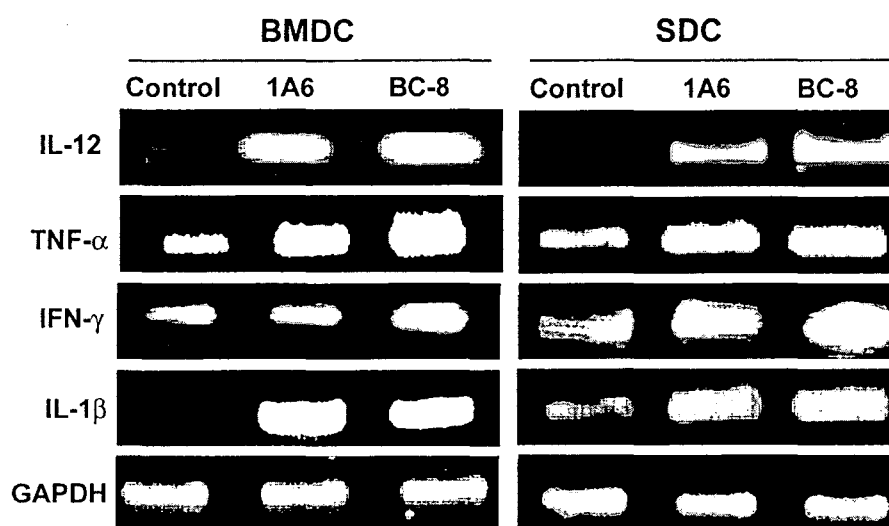
Figure 3.17

1A6 induces synthesis of immunoregulatory cytokines. Attached BMDCs and SDCs were incubated with mAb1A6 (50 µg/ml) or fixed BC-8 cells (25:1 ratio) for 24 h, after incubation with 1A6 and fixed tumor cells, total RNA was isolated from attached DCs, and subjected to RT-PCR with IL-12, TNF-α, IFN-γ and IL-1β specific primers. GAPDH signals were also amplified from same experimental samples to confirm equal loading of cDNA template.

A

Control   Isotype   1A6

LogFl$_2$H (iNOS)

Log Fl$_1$H (B7-2)

B
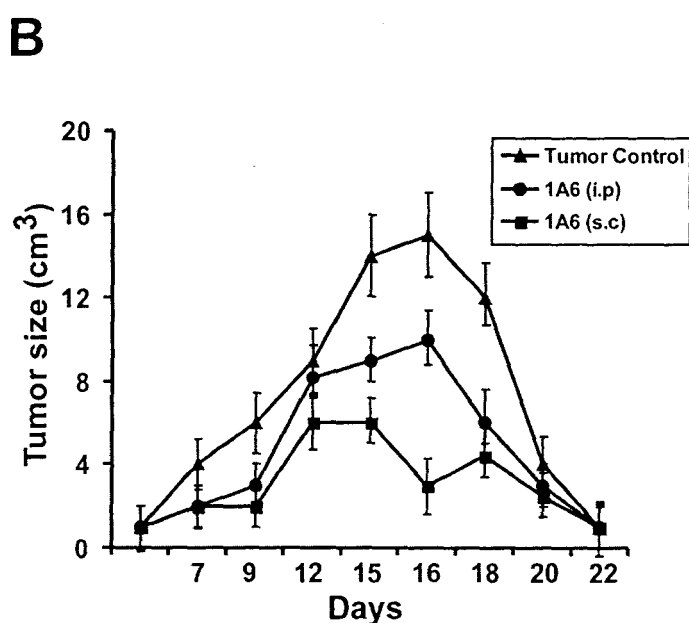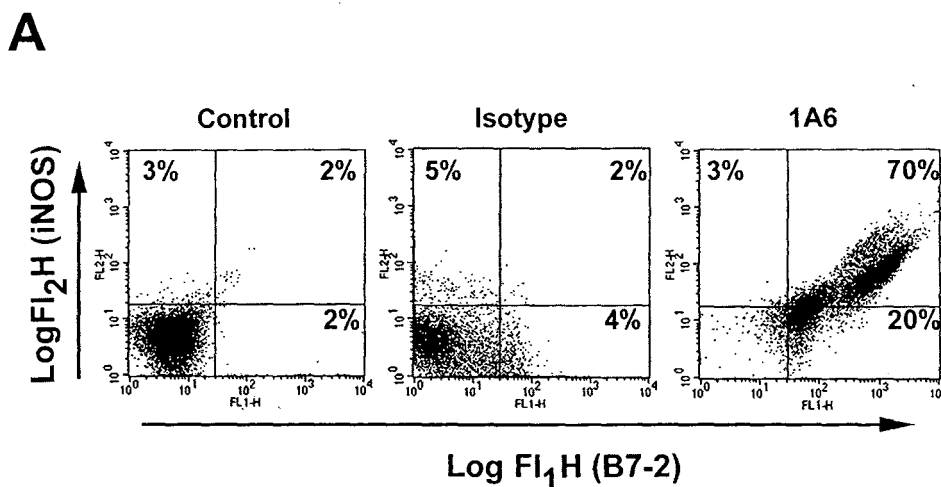
Figure: 3.18

A. *In-vivo* analysis of the activation and maturation of SDCs upon mAb1A6 injection. 1A6 (1mg/ml-PBS/rat) and isotype mAb was injected (i.p.) in wistar rats, SDCs were isolated from respective animals and fixed in 1% formaldehyde. Fixed SDCs were probed with rat anti B7-2 Ab and mouse anti-iNOS Ab. Anti-B7-2 Ab was probed with anti-rat alexa-488 (Fl$_1$H signal) and anti-iNOS Ab with anti-mouse alexa-594 (Fl$_2$H signal) simultaneously. Double staining of B7-2 and iNOS was analysed by FACS. Numbers in the figure denote the % positive population in respective quadrants.

B. Tumor growth and regression kinetics in rats challenged with AK-5 tumor cells (s.c), and co-administered with 1A6 either i.p or s.c mode (s.c. injection of 1A6 was given in the proximity of tumor site). Male Wistar rats (4-6 weeks/5 animals per group) were challenged with AK-5 tumor cells s.c. (3 x 10^6 cells/animal) and were given 9 Ab-injections (1A6 1mg/ml, i.p. or s.c. as indicated in figure) in other group of AK-5 transplanted tumor on alternate days. Tumor growth (size) was monitored on different days in 6 animals per group.

A

B

Days
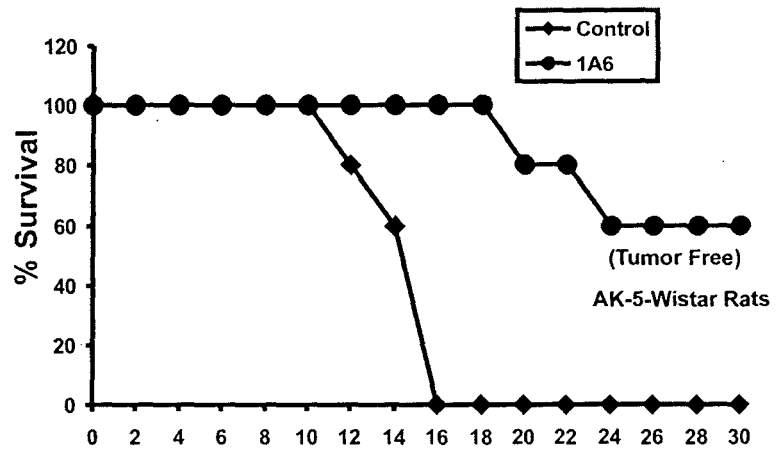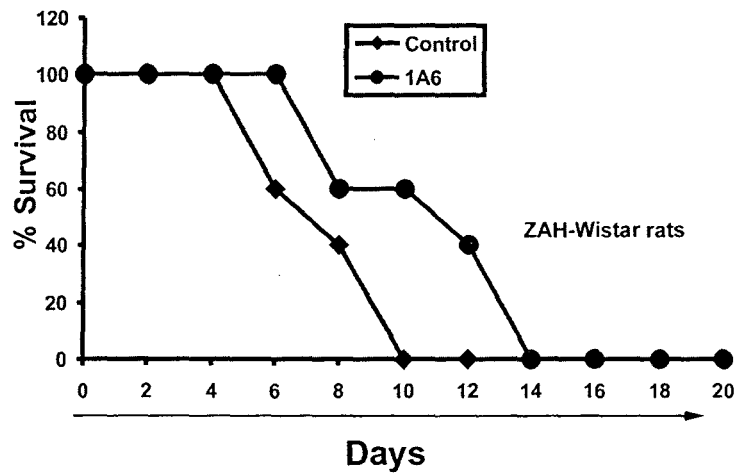
Figure 3.19

A. Effect of 1A6 on rats challenged with AK-5 tumor (i.p., 4x 10^6 cells/rat). Rats were given 3 prior injection of 1A6 (1mg/ml/rat) before transplantation of AK-5 cells and 9-1A6 injections after transplantation. Host death was monitored regularly, no tumor cells were found in 60% of rats, which was confirmed by the examination of the ascitic fluid.

B. Effect of 1A6 on rats challenged with ZAH tumor (i.p., 2x 10^7 cells/rat). Rats were given 2 prior injections of 1A6 (1mg/ml/rat) and challenged with ZAH hepatoma upon 1A6 coadministration (6 injections). Animals were routinely monitored for host death and tumor growth. A prolonged survival of rats was found in 1A6-injected animals. 10 animals per group were used for this study.

3.3 DISCUSSION

The interaction between NKG2D/NKR-P2 and its ligands is involved in anti-tumor immune responses, and the significance of this interaction is well studied for NK and CD8^{+} T-cells (Lanier, 2005b). To generate specific anti-tumor immune responses, DCs directly kill the tumor cells and present the tumor associated processed Ags upon maturation (Nouri-Shirazi et al., 2000). We have recently shown the direct killing of AK-5 histiocytoma by DCs (Alli et al., 2004).

A prerequisite for successful treatment in cancer therapy is the establishment of innate and adaptive anti-tumor immune responses; in this regard immunotherapy is an attractive approach. Because of DC's unique properties, much attention has been directed towards the use of DCs in the treatment of cancer. Our focus on immunotherapy involves the use of agonistic mAbs to activate the effector DCs upon NKR-P2 cross-linking. The absolute regulation of NK cell mediated anti-tumor immune response requires a balance between activating and inhibitory receptors whereas *in-vitro* activation of DCs with fixed tumor cells demonstrates that NKG2D ligand on tumor cells unconditionally activates NKG2D/NKR-P2 harboring DCs. NKG2D ligand^{+} tumor cells create a hostile tumor microenvironment in the proximity of DCs. The similar hostile conditions could exist *in-vivo*, when DCs migrate towards the tumor site. Usually iDCs are present at the periphery of tumor, and after interaction (Receptor-Ligand) with tumor they execute the effector function. Our *in-vitro* coculture assay (Fixed Tumor: Live DCs) confirms the natural access of ligand to DCs, which is critical for access of ligand on tumor cells. *In-vivo* being a complex environment, complete access of NKR-P2 ligand could be hindered by various factors. 1A6 mediated activation mimics a condition where easy accessibility of ligand was provided to DCs to mount the enhanced anti-tumor responses *in-vivo*. In the present study we show the efficacy of an agonistic anti-NKR-P2 mAb, which mimics NKR-P2 ligand and successfully generates protective immune responses through DCs.

Since DCs are a heterogeneous population and their functional criteria are dependent on their anatomical and differential maturation status (Gad et al., 2003), we have shown the presence of NKR-P2 on *in-vitro* generated BMDCs, which generate both, lymphoid and myeloid lineage of DCs as well as on DCs isolated from peritoneal cavity and spleen. Recently, significance of APCs in the regression of s.c. transplanted AK-5 tumor was demonstrated (Mitra et al., 2004). The efficiency of immunotherapy against tumors depends on the migratory properties of the effector immune cells. DCs are well

known for its early migration capacity towards the tumor. The slow growth of transplantable tumors in our experiments illustrates the faster and potent action of DCs. Byrne et.al have also shown that DCs rather than MHCII^{high} Mϕ are associated with skin tumor regression (Byrne and Halliday, 2003). Our data related to the activation of peritoneal DCs through mAb1A6 provides an insight into the competent mode of resident DCs activation over intravenous DCs injection since they are rapidly sequestered by lung Mϕ and are required in large numbers for immunotherapy. Intraperitoneal mode of DCs activation with 1A6 also enhances the activation of splenic DCs, which otherwise could not be achieved up to similar levels *in-vitro*. However, activation of splenic DCs with 1A6 substantiates the stable pharmacokinetic nature of 1A6 in the plasma, which is generally endowed with long half-life. DCs activated with 1A6 produce NO. *In-vitro*, iNOS induction in DCs has been demonstrated with HSPs (Panjwani et al., 2002), IFN-γ, endotoxins (Lu et al., 1996) and upon CD40 ligation (Caux et al., 1994). TNF/iNOS producing subset of DCs are also reported, which encounter invading pathogens (Serbina et al., 2003). Enhanced NO production is reported in mouse thymic DCs in response to self and allo-antigens (Aiello et al., 2000). Mouse BMDCs have also been shown to perform tumoricidal action through NO (Shimamura et al., 2002). NO is a well-known tumoricidal molecule that executes tumor cell killing by down regulating cyclin D1, inhibition of vital enzymes essential for tumor growth and through the activation of caspases (Bogdan, 2001; Farias-Eisner et al., 1994). 1A6 activated BMDCs and PDCs produce cytotoxic NO *in-vitro*, whereas SDCs showed enhanced NO production under *in- vivo* conditions. NO production from SDCs upon *in-vivo* administration of 1A6 suggests that SDCs are not resistant to NO activating signals, but do get activated with an unknown molecular signal. The secretion of NO by *ex-vivo* DCs appears to be highly variable (Powell et al., 2003). The *in-vivo* and *ex-vivo* differences reflect different lineages or different activational requirements of DCs. However *in-vivo* administration of 1A6 activated both peritoneal as well as splenic DCs, which exerted strong cytostatic action on BC-8 tumor cells *in-vitro*. 1A6 mediated NO synthesis by BMDCs, PDCs, and SDCs proves the lineage independent universal agonistic nature of the 1A6 monoclonal.

Upon NKR-P2 cross-linking by 1A6, DCs enhances NO mediated cytostatic and cytotoxic effects on tumor targets as characterized by DNA degradation and Annexin V staining of tumor cells. Recently NO has also been shown to confer therapeutic activity to DCs and a combinational immunotherapeutic approach has been proposed through DCs after NO mediated pre-sensitization of tumor (Perrotta et al., 2004; Wong et al., 2004; Yang

et al., 2005b) In this context, 1A6 mediated DCs activation is important since NO produced by the activated DCs acts as natural sensitizer to tumor cells and also promotes DCs mediated tumor cell death.

NKR-P2/NKG2D recognizes its ligand and activates the immune cells. Our data shows involvement of p38 MAPK, PI3K and EKR1/2K pathways in 1A6 mediated DCs activation. Both iNOS induction and maturation markers were significantly downregulated by inhibiting these pathways. Recently iNOS has also been shown to regulate DCs maturation process by inhibiting caspase-like activity of iDCs (Wong et al., 2004). PI3K, ERK1/2 inhibitors, EGTA, H7 and genistein caused significant reduction in NO synthesis in 1A6 stimulated DCs. These observations confirm the involvement of protein tyrosine kinase; protein kinase C and MAPK pathway in NKR-P2 mediated signaling and also proves the comparable efficiency of 1A6 with NKR-P2 ligand on DCs.

NF-κB activation plays a pivotal role in iNOS induction and DCs maturation, thus governs both innate and adaptive immunity. Cytotoxic NO production after 1A6 treatment represents strong induction of iNOS and iNOS induction is reported to be regulated by NF-κB activation. Furthermore, NF-κB acts as a potential transcription factor in the initiation of MHCII, CD86, and CD80 upregulation, as well as in IL-12 and TNF-α production (Bonizzi and Karin, 2004; Xie et al., 1994; Yoshimura et al., 2001). We have observed degradation of IκB and translocation of NF-κB upon 1A6 cross-linking. NF-κB translocation to the nucleus has also been observed upon NKG2D ligand stimulation of NK cells (Regunathan et al., 2005). Thus NF-κB activation confirms it to be the key executioner of activation upon NKR-P2 cross-linking in DCs also.

NKR-P2 is a maturation-associated receptor as its upregulation has been detected upon DCs activation with fixed BC-8 tumor cells, agonistic mAb1A6 and also in DCs obtained from tumor bearing animals (Alli et al., 2004). NKR-P2 engagement also enhanced upregulation of MHC II, CD86 and CD1a, which are involved in Ags presentation function of DCs. 1A6 activated BMDCs and SDCs were also able to cause T cell proliferation in autologous MLR assays. However, SDCs were slightly more efficient which could be due to their maturational status. In readout, the endocytic capacity of 1A6 stimulated BMDCs and SDCs was significantly inhibited upon 1A6 stimulation, which also suggests it to be a potent maturational stimulus. We have also detected homotypic aggregation of BMDCs and SDCs upon 1A6 stimulation. Cellular aggregation in response to surface Ags-signaling is a regulated process and important for cell-cell interaction. Previously we have shown relocalization of NKR-P2 upon 1A6 cross-linking in splenic

DCs (Alli et al., 2004), which suggests that DCs form strong cellular interactions with tumor cells during NKR-P2 interaction with its natural ligand on tumor cell surface. Similar clustering events have also been observed upon MHC II and CD43 cross-linking (Corinti et al., 1999; Lehner et al., 2003) that could drive the formation of "DCs-T" cell cluster to provide efficient stimulatory signal.

1A6 treatment also induces DCs to produce higher-level of proinflammatory cytokines TNF-α, IL-1β, IFN-γ, and IL-12. Previously, we have shown enhanced production of IL-12 from DCs with fixed AK-5 cells. IL-12 produced upon DCs activation also augments NK cell cytotoxicity which inturn induces NK cell function (Alli et al., 2004).

Faster regression of AK-5 tumor was observed upon s.c. or i.p. administration of mAb1A6 in tumor growing rats suggesting the enhancement of DCs mediated effective immune response. Increased survival of tumor bearing hosts at low and high dose of tumor demonstrates the capacity of DCs to mount immune responses at highly critical stage. Recently, potent anti-tumor immunity has been attained upon intratumoral targeting of DCs (Furumoto et al., 2004). Previously, we have reported that DCs isolated from 1A6-injected animals exhibit enhanced apoptosis in BC-8 tumor cells in-vitro (Alli et al., 2004). Both iNOS and maturation status of splenic DCs were enhanced upon 1A6 administration in-vivo, which also suggests the possible direct role of DCs in tumor regression in-vivo along with other effector cells. Thus, we show that the agonistic mAb1A6 possess the potential to enhance DCs mediated anti-tumor activity. Recently an analogous approach of targeting DCs through NKG2D ligand has also been reported, which generates efficient anti-tumor immune response (Groh et al., 2005).

In summary, our results demonstrate that anti-NKR-P2 mAb pulsed DCs induce both innate and adaptive immune responses. This study demonstrates specific targeting of the tumor recognition receptor on DCs, which only operates under stress conditions and recognizes multifarious ligands, displaying its efficiency in a broad range. Our results also suggest an important role for NKR-P2 expressed on DCs in stimulating the anti-tumor immune response and the potential use of a mAb against NKR-P2 in cancer immunotherapy.

CHAPTER IV

The Ischemia Responsive Protein 94 Activates Dendritic cells Through NKR-P2 Leading To Their Maturation

4.1 INTRODUCTION

Dendritic cells (DCs) are unique subset of leukocytes, specialized for Ag processing and presentation (Banchereau and Steinman, 1998). DCs are well characterized for their important role in regulating T-cells against tumor (Lanzavecchia and Sallusto, 2001).

DCs infiltrate in tumors in high number (Preynat-Seauve et al., 2006), kill tumor cells and present their Ags to elicit tumor specific immune responses to mediate tumor rejection (Nouri-Shirazi et al., 2000; Furumoto et al., 2004). The recognition of 'tumor Ags' is the first crucial step in both innate and adaptive anti-tumor immunity. The direct contact mechanism must include the recognition of a 'distinctive element' on target cells by corresponding receptor on DCs to induce direct cytolysis (Sashchenko et al., 2007). Hence the mechanism behind direct killing of tumors by DCs has attained greater attention.

DCs recognize tumors through TRAIL, FasL and lymphotoxin-$\alpha_1\beta_2$ receptors (Lu et al., 2002). Cytotoxic rat DCs also express NKR-P1 (Trinite et al., 2000) and NKR-P2 (Alli et al., 2004) for tumor recognition. NKR-P2/NKG2D is a disulfide linked type II c-type lectin like receptor expressed also on NK cells, CD4+, CD8+$\alpha\beta$, $\gamma\delta$+ T cells (Berg et al., 1998), rat CD4$^+$/CD8$^+$ MOs and Mϕ (Baba et al., 2006), mouse IKDCs (Taieb et al., 2006; Chan et al., 2006), and human myeloblastic KG1a cells (Guilloton et al., 2005), which show a versatile expression of NKG2D on immune cells.

NKG2D/NKRP-2 interacts with multiple ligands, which are usually upregulated on tumors, stressed cells and mounts anti-tumor immune response. A range of NKR-P2/NKG2D ligands have been identified which include MICA/MICB, ULBPs proteins in humans; Rae1, H60 and MULT1 in mice, exhibiting an enigmatic diversity of recognition components for NKG2D. NKG2D ligand transduced cells are vigorously rejected by syngeneic animals, which display an effective role of ligands in anti-tumor immune response (Raulet, 2003). NKG2D interacts with dissimilar ligands by induced fit mechanism and displays a 'degenerate recognition system'. Biophysical studies have explored the H-bonds, hydrophobic interactions and salt bridges as crucial components, which contribute to maintain the overall shape complementarity for induced fit recognition of NKG2D ligands (Strong and McFarland, 2004; O'Callaghan and Jones, 2003). NKG2D uses dimeric surface for ligand binding, which is a characteristic of c-type lectin like domain and associates with multiple adapter units to endow high sensitivity (Garrity et al., 2005; Sun, 2003).

Crystal structure of human NKG2D and *in-silico* and *in-vitro* alanine-scanning mutagenesis analysis of the complex interfaces indicate that NKG2D recognition degeneracy

cannot be exclusively explained by a classical 'induced-fit mechanism'. Refined structural analysis also suggests that divergent ligands utilize different strategies to interact with structurally conserved elements of the consensus NKG2D binding site (O'Callaghan and Jones, 2003).

AK-5 is a highly immunogenic MHCI histiocytoma and regresses spontaneously upon s.c. transplantation in syngenic animals whereas it kills 100% hosts upon i.p. transplantation (Khar et al., 1998). Previously it was demonstrated that anti-NKR-P2 mAb enhances cytotoxic action of DCs, and soluble NKR-P2 protein binds on AK-5 surface in a patchy pattern (Alli et al., 2004). Fixed AK-5 cells also induce DCs maturation (Alli and Khar, 2004), These findings prompted us to identify a putative NKR-P2 ligand on AK-5 cell surface that may interact with NKR-P2 on DCs. During our screening for NKR-P2 ligand on AK-5 cells, we have identified that a 110-kDa heat shock protein namely ischemia responsive protein 94 (Irp94) acts as an interacting partner for NKR-P2. Irp94 upregulation is reported in rat brain under ischemic conditions and in endoplasmic reticulum with stress inducing drugs (Yagita et al., 1999; Kim et al., 2001). Irp94 is a member of 110-kDa hsp family and a homolog of mouse APG-2 and human hsp70RY. Irp94 is >90% identical to APG-2, hsp70RY and ~60% homologous to other hsp110 family members, and contains hsp70 signatures at c-terminus subdoamin. At c-terminus from amino acid (AA) 616 to 699 and from AA 701 to 794, Irp94 shows homology with 'hsp70 superfamily'. In general HSP110 family members are related in sequence and structure to the hsp70 family and together referred as the "hsp70 superfamily" (Easton et al., 2000). Theriault, et al. have recently shown the interaction of NKG2D to hsp70, which also confirms the broad range of HSPs recognition by NKG2D/NKR-P2 (Theriault et al., 2006).

Several studies have suggested that HSPs act as danger signals and mount immune response by activating APCs through specific receptors (Srivastava, 2002a; Srivastava, 2002b). A broader search through literature revealed that surface expression of HSPs including hsp70 is a widespread phenomenon on tumor cells [reviewed in general introduction 1.5.1]. At the same time surface expression of HSPs on tumor cells gives an insight into direct interaction between tumor and immune cells (Multhoff, 1997; Multhoff et al., 1998; Multhoff et al., 1995b; Multhoff and Hightower, 1996; Gastpar et al., 2005; Kleinjung et al., 2003; Steiner et al., 2006; Becker et al., 2004). It was previously demonstrated that cytotoxic NK cells recognize hsp70 as tumor specific recognition structure (Multhoff et al., 1997). Gastapar et al. have demonstrated that tumor specific surface hsp70 induces cytolysis and migration of human NK cells, in their study hsp70 epitope exposed to

the extracellular side of tumor was also identified "TKDNNLLGRFELSG (TKD; aa 450–463)" which is part of the c-terminal domain of hsp70 (Gastpar et al., 2004). Although the immunological role of membrane-bound HSPs on tumor cells appears apparent, the mechanism of transport to the plasmamembrane, the membrane anchorage is enigmatic (Schmitt et al., 2007).

In the present study, we have demonstrated NKR-P2 expression on rat iBMDCs and its upregulation upon maturation. Irp94 surface expression was confirmed by immunostaining with anti-Irp94 mAb on AK-5 cells and on tumor cells of diverse origin. Irp94 specifically binds to NKR-P2 expressing DCs, NK cells and T cells. We have studied the interaction of recombinant Irp94 with NKR-P2 on DCs and shown that DCs stimulatory capacity resides in the COOH-terminus sub-doamin of Irp94, whereas NH_2-terminus ATPase subdomain is unable to stimulate DCs. Irp94 specificity to NKR-P2 was assessed as pull down product with ECD (extracellular domain) of NKR-P2. Specific binding of fluorescent Irp94 with NKR-P2 on DCs also corroborated its specificity and functional capability. When stimulated by Irp94-NKR-P2 interaction, BMDCs produce NO in a Ca^{++} dependent manner and induce apoptosis in AK-5 cells. Similarly, Irp94-NKR-P2 stimulation induces cytokine release and maturation of BMDCs, which is crucial for an adaptive immune response. Irp94-NKR-P2 induced signal transduction was investigated with the help of various pharmacological inhibitors that indicated involvement of MAP kinases in signaling pathways. NF-κB translocation appears to be a crucial downstream target of Irp94 mediated activation. Differential surface expression of Irp94 correlates with the immunogenicity of tumor in-vivo. Thus Irp94 acts as a novel ligand for NKR-P2 on DCs, which is different from the known ligands of NKG2D.

Given the promising role of NKR-P2 in DCs mediated tumor cell killing, the present study was intended for a better understanding of the tumor recognition component (NKR-P2 ligand) by DCs. Our study demonstrates for the first time, the role of hsp110 family protein as a functional ligand of tumor recognition receptor and puts forth its significant role in DCs mediated anti-tumor immune response.

4.2   RESULTS

4.2.1   Irp94 and its interaction with NKR-P2

To identify the interacting ligand (s) for NKR-P2, AK-5 cDNA expression library was screened and Irp94 (a110-kDa heat shock family protein) was found to be positive. The

interaction of Irp94 with soluble recombinant NKR-P2-GST fusion protein was confirmed in multiple rounds of secondary and tertiary screening with GST alone as negative control. In order to find out the interacting segment in full length Irp94, we generated two deletion constructs, namely COOH-terminal subdomain (cIrp94, aa. 493-840) and NH$_2$-terminus ATPase domain (nIrp94, aa.1-175), and full length Irp94 (FlIrp94, aa. 1-840) **(Fig. 4.1 A).**

The interaction between Irp94 and NKR-P2 ECD was confirmed in an *in-vitro* pull down assay. Equimolar ratio of soluble recombinant NKR-P2-GST or GST alone was immobilized on glutathione agarose beads and the interaction with pure soluble recombinant Irp94-His tag protein was studied. The assay demonstrated specific interaction of NKR-P2 and Irp94 by pulling down Irp94 along with recombinant ECD of NKR-P2, over the GST and bead alone as negative controls **(Fig. 4.1B).**
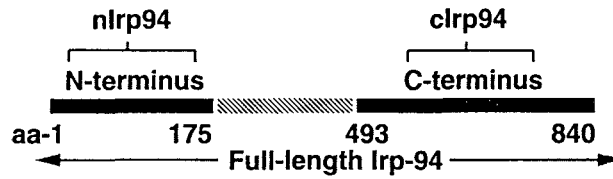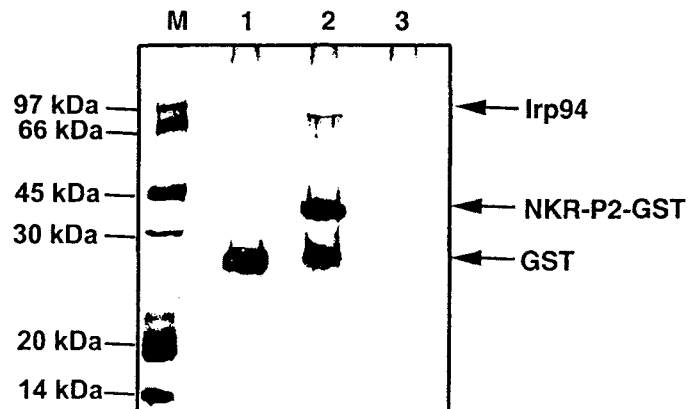
We fluorescinated cIrp94 (FITC-cIrp94) with FITC coupling protocol, and specificity of Irp94 binding to NKR-P2 was confirmed by staining with FITC-cIrp94 on ectopically expressed NKR-P2 on CHO cells **(Fig. 4.2A).** Further we checked the binding of soluble NKR-P2/NKG2D on ectopically expressed Irp94 and observed enhanced binding of soluble NKR-P2 on ectopically expressed Irp94. Additionally enhanced binding of NKR-P2 was also found on ectopically expressed hsp70.Cκ **(Fig4.2B).**

4.2.2 Expression and distribution pattern of Irp94

A mAb (2F4) against cIrp94 was generated and specificity of 2F4 mAb was established by competitive binding of 2F4 on AK-5 cells, which shows significantly lower binding upon preneutralization with recombinant cIrp94 **(Fig 4.3).**

AK-5 tumor cells were stained with mAb 2F4 to depict the surface expression of Irp94. mAb 2F4 stained in a patchy localized or a patchy non-uniform pattern on the plasmamembrane of AK-5 cell surface **(Fig. 4.4A).** To depict the cellular localization of Irp94 in AK-5 cells, a thin section confocal scanning of AK-5 cells was performed after staining with mAb 2F4; Fibrous and granular staining pattern of Irp94 was found at the perinuclear region suggesting its localization in endoplasmic reticulum and Golgi complex intracellularly. Furthermore, membrane localization of Irp94 was evident in middle section **(Fig. 4.4 B).**

Surface expression of Irp94 was also confirmed on AK-5 cells after immunostaining with polyclonal anti-Irp94 Ab and with mAb 2F4, both of which were generated against cIrp94 **(Fig. 4.5A).** Irp94 expression was also detected in several tumor cell lines of murine and human origin as confirmed by the semiquantitative RT-PCR analysis **(Fig. 4.5B).**

A

nIrp94

N-terminus

cIrp94

C-terminus

aa-1   175   493   840

Full-length Irp-94

B

M   1   2   3

97 kDa —
66 kDa —   ◄— Irp94

45 kDa —   ◄— NKR-P2-GST
30 kDa —   ◄— GST

20 kDa —

14 kDa —

Figure: 4.1

A. Domain configuration of the expressed cDNA deletion constructs of full length Irp94: C-terminus sub-domain (AA: 493-840); cIrp94. N-terminus ATPase domain (AA: 1-174) nIrp94 and full length (AA:1-840); FIIrp94 were generated as deletion constructs.

B. Soluble Irp94 protein specifically interacts with soluble NKR-P2: GST Pull down assay product of NKR-P2 (Total extracellular domain;ECD),where Irp94 is pulled down along with soluble NKR-P2 as shown in lane 2. Lane1 represents GST and lane 3 represents immobilized agarose beads.

A

FITC-cIrp94(LogFI₁H)
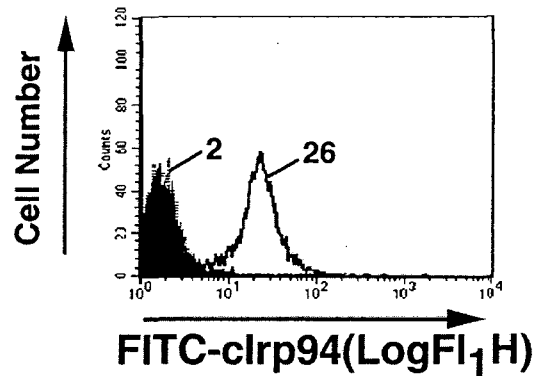
B
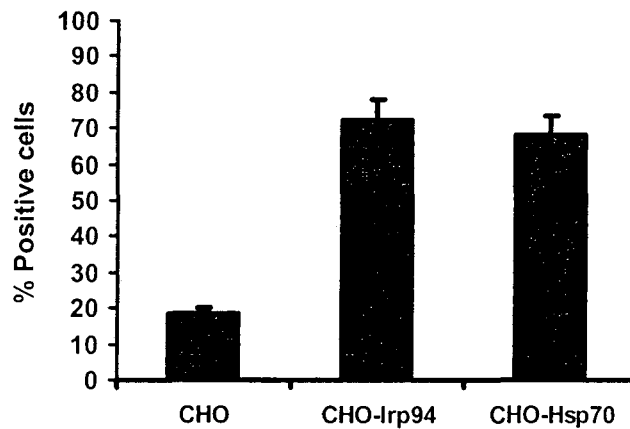
Figure 4.2

A. FACS analysis of FITC-cIrp94 binding on ectopically expressed NKR-P2 on CHO cells: Live CHO cells (dotted line) or CHO transfected with full length NKR-P2 cDNA (bold line) were stained with FITC-cIrp94 and analysed by FACS. Binding intensity of FITC-cIrp94 on NKR-P2 transfected CHO cells (MFI) is represented as numbers in the figure. Filled histogram denotes autofluorescence of CHO cells.

B. Full length Irp94 and Hsp70cκ cDNA were transfected in CHO cells and stable clones were selected in G418. Binding of soluble NKR-P2/ NKG2D (100μg/ml) was assessed on transfected cells. An enhanced binding of soluble NKG2D protein was found on Irp94 transfected CHO cells. Similarly enhanced binding of sNKG2D was also observed on Hsp70 transfected clones. Binding of soluble NKG2D was assessed by FACS and shown as % positive cells.
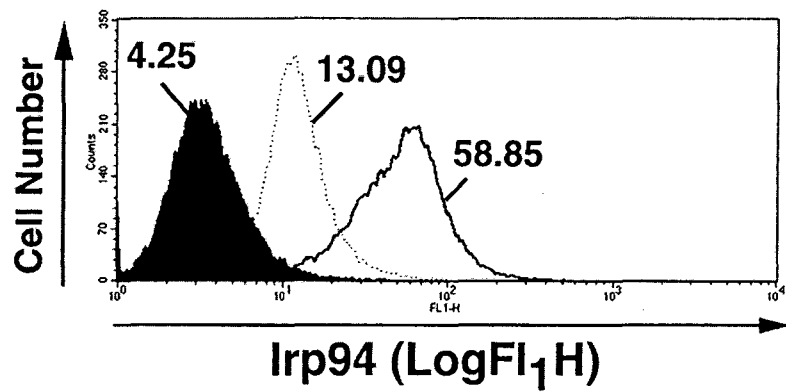
Figure 4.3

Specificity of mAb 2F4: Fixed AK-5 cells were incubated with mAb 2F4 (Bold line), or with mAb 2F4 pre-incubated with recombinant cIrp94 ($50\mu g/ml$, 2h, $4^{0}C$) broken line. Cells were stained with anti-mouse alexa-488 and analysed by FACS. MFI is represented in numbers along with marked histogram. Filled histogram shows secondary Ab control.

Figure 4.4

A. Surface staining of Irp94 on AK-5 cells: AK-5 cells were stained with mAb 2F4 (anti-cIrp94) followed by FITC-conjugated secondary Ab (alexa-488): Upper panel shows localized surface staining (green); Lower panel shows non-unifrom patchy staining pattern (green); Nucleus (N) was stained with propidium iodide (red). Left panel: Transmission image of cells; Middle panel: Surface staining; Right panel: Surface staining overlaid on the transmission image (Maginification: 100X).

B. Confocal images of Irp94 at perinuclear region along with surface of AK-5 cell: AK-5 cells were permeabilised with methanol: acetone (1:1) for 30 min at 4⁰C. Washed AK-5 cel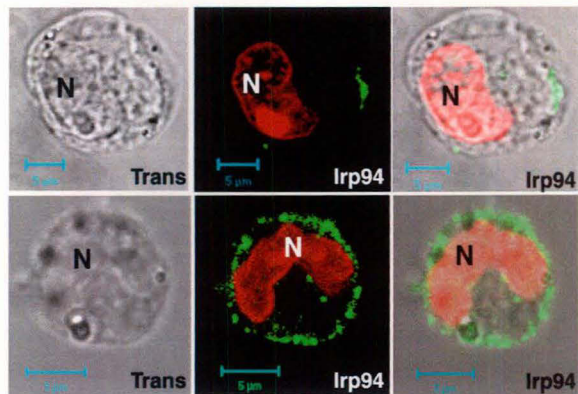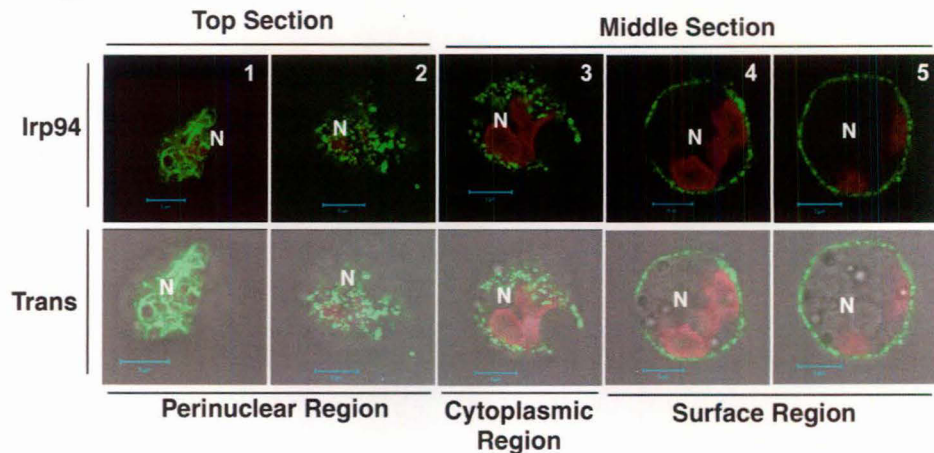ls were probed with mAb 2F4, followed by FITC-conjugated secondary Ab (alexa-488). Nucleus (N) was stained by propidium iodide (red). Panels 1 and 2 show perinuclear-staining intracellualry, panel 3 shows cytoplasmic staining, panels 4 and 5 show staining on plasmamembrane. (Magnification: 63X;Section thickness:0. 5μ). Corresponding transmission images (superimposed) are shown in lower panel.

A

Irp94 (LogFl$_1$H)
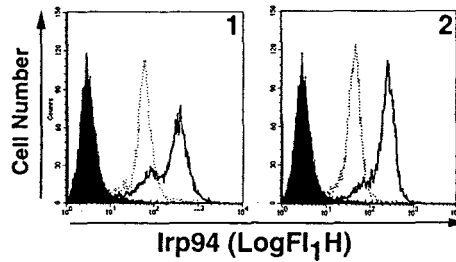
B
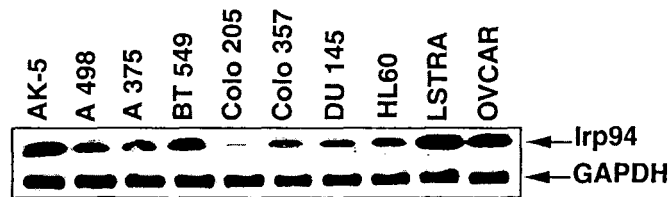
Figure 4.5

A. FACS analysis of surface Irp94 expression on AK-5 cells: AK-5 cells were stained with mouse polyclonal anti-Irp94 serum and with mAb 2F4 against Irp94. Panel 1: live AK-5 cells (Surface Irp94); panel 2: permeabilised AK-5 cells. (Filled histogram: secondary Ab controls (alexa-488); Open Histogram: dotted line- anti-Irp94 polyclonal Ab, Bold line–mAb2F4.

B. Semiquantitative RT-PCR analysis showing expression of Irp94 on AK-5 (rat histiocytoma), A498 (Human renal cell carcinoma), A375 (human melanoma), BT549 (breast carcinoma), Colo205 (human colon carcinoma), Colo357 (human pancreatic tumor cell), DU145 (human prostrate carcinoma), HL60 (human myeloid tumor), LSTRA (murine lymphoma) and OVCAR (human ovarian carcinoma). GAPDH control is also shown.

Being a heat shock protein, we also checked surface expression of Irp94 on AK-5 cell surface upon sublethal heat shock. As speculated, surface expression of Irp94 increases significantly upon sub-lethal heat shock suggesting a heat inducible translocation of Irp94 on the plasmamembrane **(Fig. 4.6)**.

In addition to AK-5 cells, surface expression of Irp94 was also analysed on a panel of tumor cell lines by live cell staining with mAb 2F4 and most of the tumor cell lines were found to express Irp94, whereas low level of expression was observed on rat peritoneal Mϕ, rat fibroblast cell line F111, rat PBMCs but not on splenocytes and BM cells **(Fig. 4.7)**. High surface expression of Irp94 on AK-5 and other tumors but not on normal cells suggested that surface Irp94 may acts as tumor recognition structure.

4.2.3 Surface expression of Irp94: An enigmatic mechanism

Irp94 neither contains the membrane localization signal nor does it have GPI anchorage properties hence its presence in plasmamembrane of tumor cell is enigmatic. Transcriptional inhibitor Actinomycin-D and translational inhibitor Cyclohexamide significantly reduced the surface Irp94, which suggests involvement of an active process for membrane localization. Treatment with BrefeldinA, (which aborts the classical anterograde transport of proteins) failed to reduce surface Irp94 suggesting a non-classical mode of Irp94 transport to plasmamembrane **(Fig. 4.8A)**.

Patchy and non-uniform Irp94 staining on plasmamembrane indicated the raft-associated expression of Irp94. However raft disintegration by cholesterol depletion from membrane by methyl-β-cyclodextrin failed to reduce surface Irp94, which confirmed a non-raft associated surface expression of Irp94 in plasmamembrane **(Fig. 4.8B)**.

Irp94 resides in ER region, and ER stress inducible drugs (H_2O_2, DTT, Tunicamycin, and MG132) significantly enhanced surface expression of Irp94 whereas brefeldin A failed to increase the surface expression. A potent proteosomal inhibitor Lactacystin reduced the surface expression of Irp94 **(Fig. 4.9)**. These results show that surface Irp94 in tumor cells is related with stress condition, yet the molecular mechanism for its presence at surface remained an enigma.

4.2.4 Irp94 stimulates upregulation of NKR-P2 on DCs

The generated BMDCs were immature as assessed by dendritic morphology and quantitation of phenotypic markers like MHC II, CD1a, B7-2, $\alpha\epsilon$-integrin and CD11c. Upon

Figure 4.6

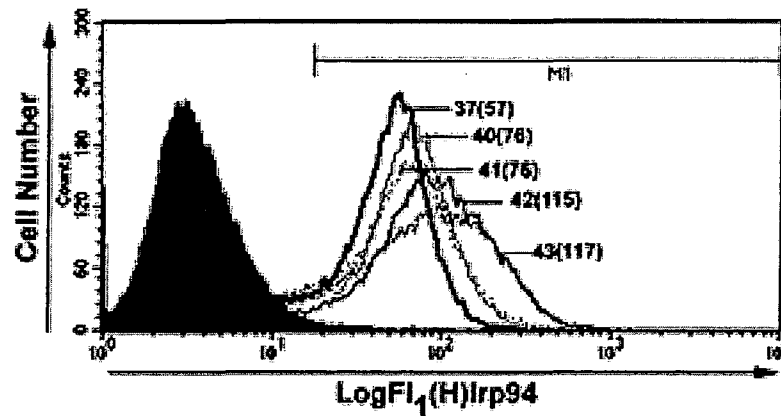Enhanced surface expression of Irp94 upon sub-lethal heat shock: BC-8 (single cell clone of AK-5) cells were subjected to heat shock (at 40^0C, 41^0C, 42^0C, 43^0C for 45 min: Recovery time after heat shock: 6 h) and the cells were stained with mAb 2F4 (Filled histogram: secondary Ab control (alexa- 488), open histogram: mAb 2F4; MFI is represented in numbers along with marked histograms.
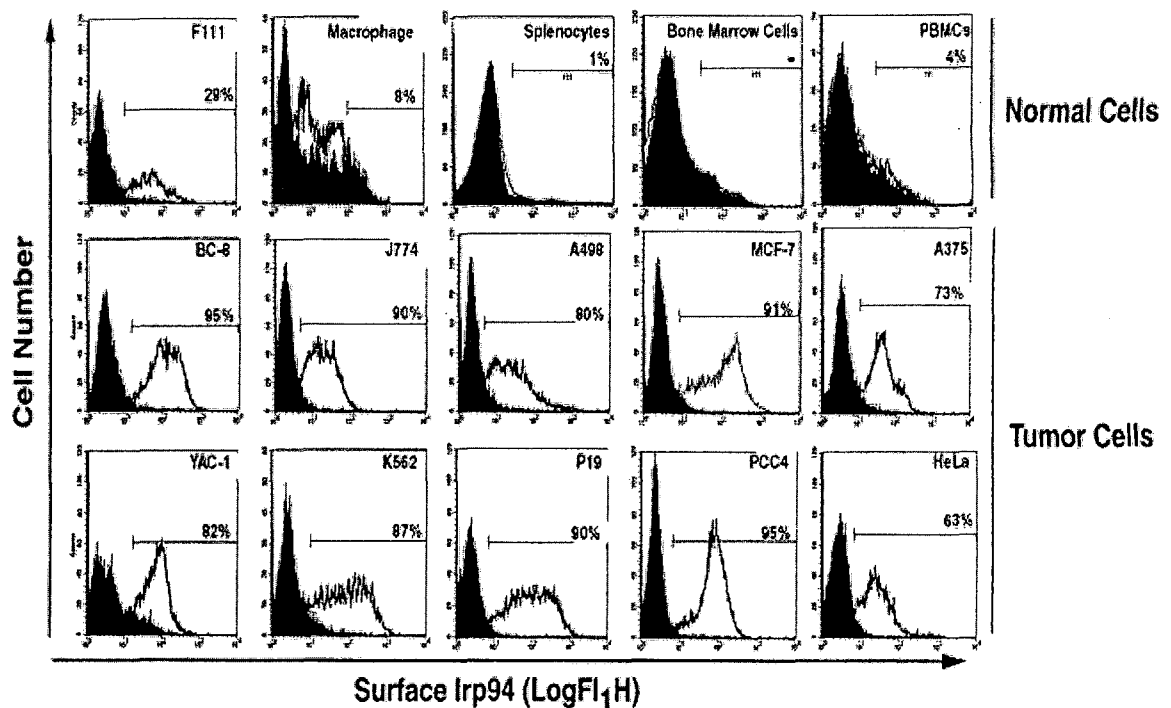
Figure 4.7

Surface expression analysis of Irp94 with mAb 2F4 on various tumor cell lines and on normal cells. Live F111 (rat fibroblast cell line), rat peritoneal macrophage, rat splenocytes, rat bone marrow cells, rat PBMCs, and live BC-8 (single cell clone of AK-5), J774 (murine macrophage tumor cell line), A498 (human renal cell carcinoma), MCF7 (human breast cancer cell), A375 (human melanoma), YAC-1 (murine lymphoma), K562 (human erythromyleoid leukemic cell line), P19 and PCC4 (mouse embryonal carcinoma), HeLa (Human cervical carcinoma). Upper panel shows surface expression of Irp94 on live normal cells; Middle and lower panels show surface staining of Irp94 on various transformed/ tumor cell lines of multiple origins.

Live cells were stained with mAb 2F4 (Open histogram) followed by staining with secondary Ab (alexa-488) (Filled histogram). One representative experiment of the two experiments is shown. Figure depicts the surface expression of Irp94 on various tumors/ transformed cell lines, but little or absence of Irp94 on the surface of normal immune cells. % Positive population (Numbers) is shown under marking M1, which depicts the positive staining in open histogram.
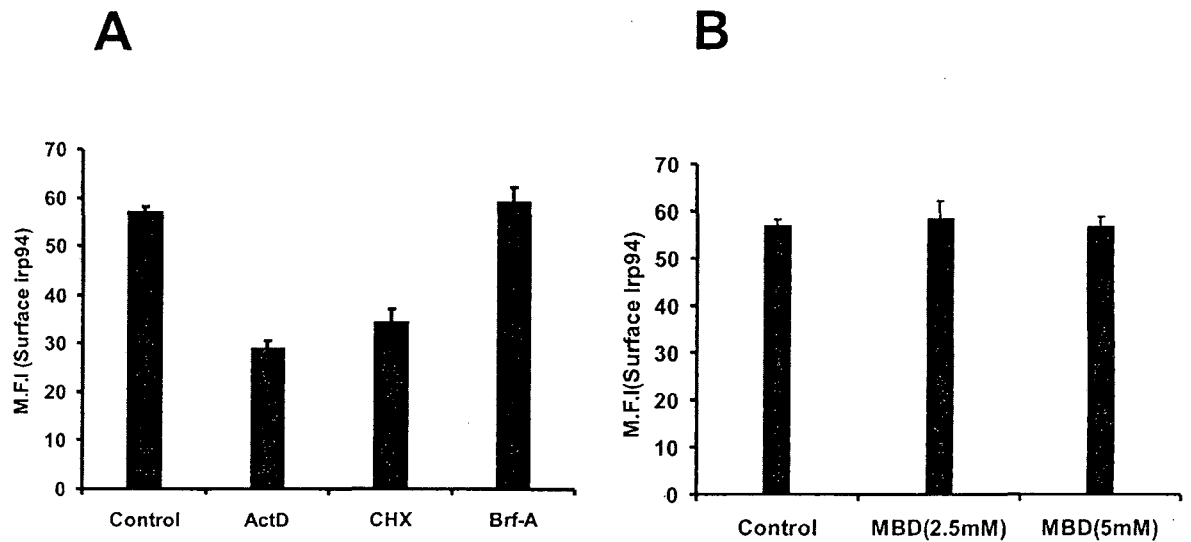
Figure: 4.8

A. Surface expression of Irp94 and its regulation. BC-8 tumor cells were incubated for 6h with sub-lethal concentration of Actinomycin-D (ActD:1µg/ml), Cyclohexamide (CHX:2µg/ml) or BrefeldinA (Brf-A:2µg/ml) and cell surface Irp94 was determined after staining with mAb 2F4 on live BC-8 cells,followed by FACS analysis.(MFI: surface Irp94 is shown in figure as bar diagram)

B. Surface expression of Irp94 is not raft-associated. BC-8 cells were incubated with sub-lethal concentrations of methyl-β-cyclodextrin for cholesterol depletion (MBD: 2.5mM, 5mM) for 1h in incomplete media (cholesterol depletion 25-30%) and washed. Afterward surface Irp94 was determined on live BC-8 cells by FACS (MFI: surface Irp94 is shown in figure as bar diagram)
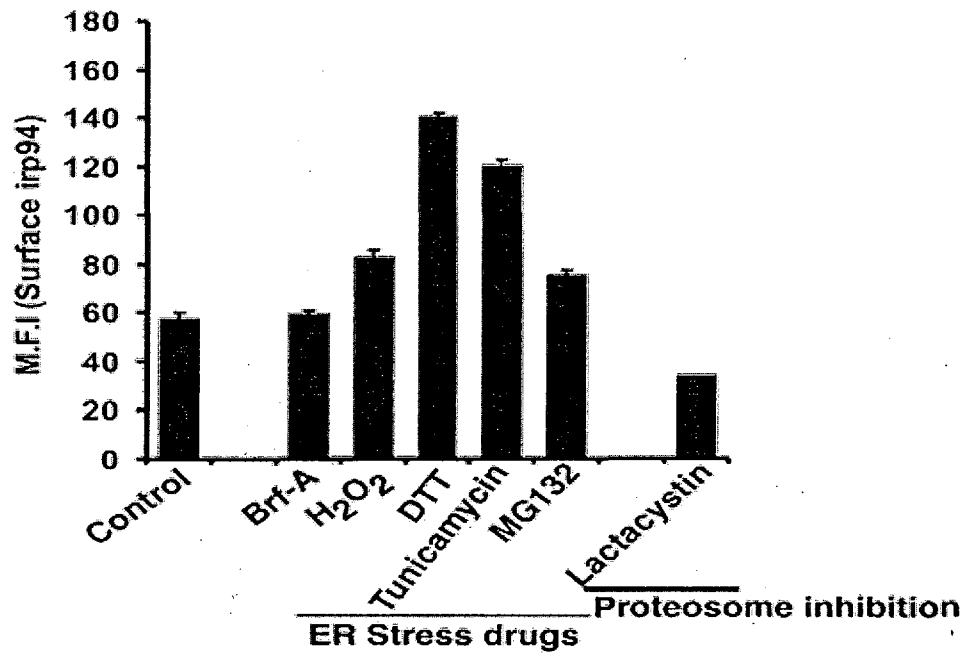
Figure 4.9

Surface expression of Irp94 upon ER stress. BC-8 cells were treated with sub-lethal concentrations of stress inducing drugs for 6h (Brf-A: $2\mu g/ml$, H_2O_2:2mM,DTT:2mM,Tunicamycin:$2\mu g/ml$,MG132:5mM, Lactacystin:$1\mu M$). Afterwards surface Irp94 was determined by FACS, by staining with mAb2F4.

LPS treatment, significant up regulation of MHCII, CD86 and CD1a takes place whereas αε-integrin and CD11c expression remain unchanged as assessed by flow cytometry **(Fig. 4.10)**.

In continuation with our earlier observations (Alli et al., 2004), where NKR-P2 expression was upregulated on activated splenic DCs, we analyzed NKR-P2 expression on immature and LPS matured BMDCs, and NKR-P2 was found to be upregulated on LPS matured BMDCs **(Fig. 4.11)**. RT-PCR analysis also correlated well with the increase in NKR-P2 levels upon cIrp94 and LPS treatment on both BMDCs and splenic DCs **(Fig. 4.12A)**. These results suggest that NKR-P2 is upregulated upon stimulus with cIrp94, on both splenic DCs and BMDCs.

In order to study NKR-P2-Irp94 interaction at the cellular level, fluorescinated cIrp94 was used and the binding of FITC-cIrp94 on immature and LPS treated mature BMDCs was analysed. In accordance with our previous observations, where NKR-P2 was upregulated on BMDCs upon LPS treatment (fig. 4.11), we observed enhanced binding of FITC-cIrp94 on LPS matured BMDCs, which were counterstained for CD11c and αε-integrin **(Fig. 4.12B)**.

4.2.5 Irp94 as an interacting ligand for NKR-P2 on DCs

FITC-cIrp94 binding was specific to NKR-P2, as judged by its binding pattern on immune splenic DCs (from AK-5 tumor bearing rat) where NKR-P2 is expressed as a raft like clustered pattern, in contrast to splenic DCs from normal rats where NKR-P2 is expressed all along the cell membrane. This typical pattern of colocalisation of FITC-cIrp94 with NKR-P2 indicates its binding specificity with NKR-P2 **(Fig. 4.13)**. In order to further establish the specificity of FITC-cIrp94 binding to NKR-P2, we performed the competitive binding assay after NKR-P2 pre-blocking with mAb1A6 and untagged cIrp94. The binding of FITC-cIrp94 was significantly inhibited upon pre-blocking NKR-P2 with mAb1A6 and cIrp94 (200μg/ml for each) **(Fig. 4.14A)**. Inhibition of FITC-cIrp94 binding was also observed on NK cells and CD8$^+$ T cells upon mAb1A6 pre-blocking, which proved specific interaction of Irp94 to NKR-P2 expressing cells **(Fig.4.14B)**. The interaction of irp94 and NKR-P2 at cell surface was also observed in DCs and AK-5 conjugate staining **(Fig.4.15)**.

4.2.6 Activation of DCs upon NKR-P2-Irp94 interaction

Since activated BMDCs are known to produce significantly higher amount of NO as compared to resting DCs, we measured NO production as a measure of iNOS activity to assess DCs activation. Day 6 iBMDCs were incubated with increasing concentrations of cIrp94 or with LPS as positive control, which led to NO secretion in a dose dependent
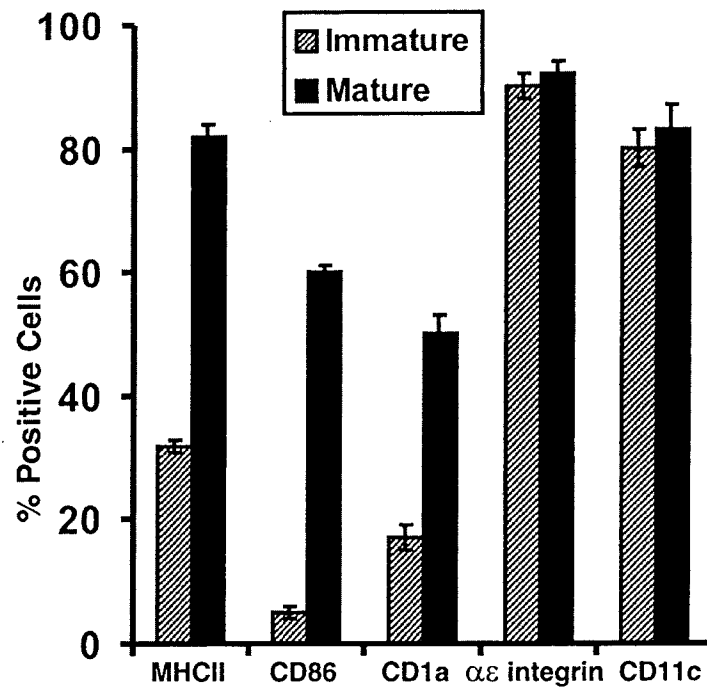
Figure: 4.10

Quantitation of phenotypic DC markers, MHC II, CD86, CD1a (Maturation markers), OX-62 and CD11c (Non-maturation markers) on immature and mature BMDCs. Upregulation of maturation markers (MHC II, CD86 and CD1a) was observed upon LPS treatment on day 6th BMDCs in comparison to untreated DCs, as detected by FACS. % Positive cells are shown for specific DC markers in bar diagram. Results shown are representative of more than five similar experiments with different sets of *in-vitro* generated BMDC.
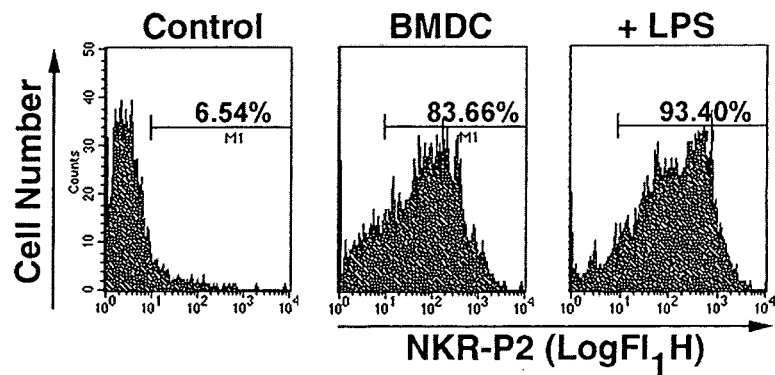
Figure 4.11

Day6 immature and LPS matured BMDCs were harvested and surface-expression of NKR-P2 receptor was analysed on FACS with mAb 1A6. Panels: secondary Ab control (left), 6th day immature (middle), LPS matured (right) BMDCs. % Positive cells are shown in numbers under the marking M1. Results shown are representative of three similar experiments with different sets of *in-vitro* generated BMDCs.
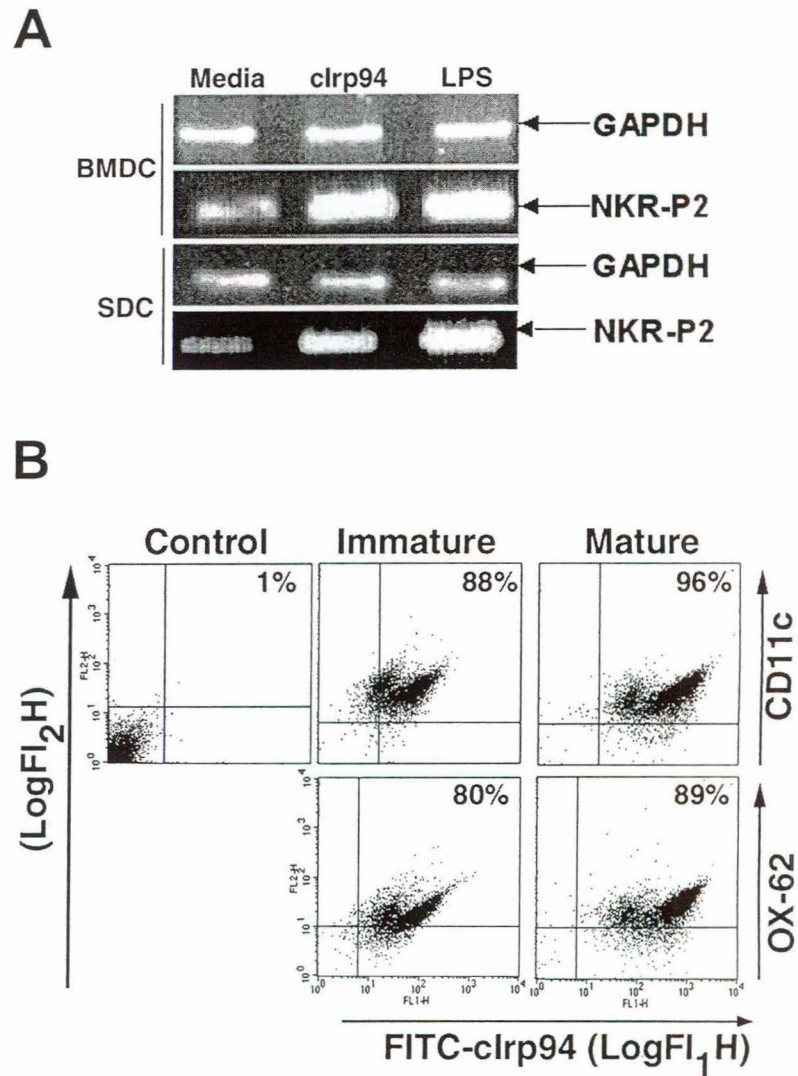
Figure: 4.12

A. RT-PCR analysis of NKR-P2 transcript expression. NKR-P2 expression increases with the activation signals viz. cIrp94 and LPS. Upper panel (BMDCs), lower panel (SDCs). RNA was isolated after 24 h stimulation with either cIrp94 or LPS and subjected to RT-PCR with NKR-P2 specific primers. GAPDH control is also shown that depicts equal loading of total cDNA as template.

B. Comparison of immature and LPS mature BMDCs with respect to binding of FITC-cIrp94. Untreated (immature) and LPS treated (mature) BMDC were stained with CD11c and OX-62 mAb followed by alexa-594 seconadary Ab. (CD11c and OX-62 are non-maturation markers) (Y-axis); CD11c and OX-62 stained cells were counterstained for FITC-cIrp94 (X-axis) binding. Increased binding of FITC-cIrp94 was observed on LPS mature BMDCs because of upregulation of NKR-P2 on mature BMDC. Numbers of % positive cells are show that depicts staining in upper right quadrant. Upper left panel show the staining of secondary Ab(alexa-594).

| Bright Field | mAb 1A6 | FITC-cIrp94 | Merge | |
| Naive SDC |
| Immune SDC |

| Bright Field | mAb 1A6 | FITC-cIrp94 | Merge | |
| Naive SDC |
| Immune SDC |
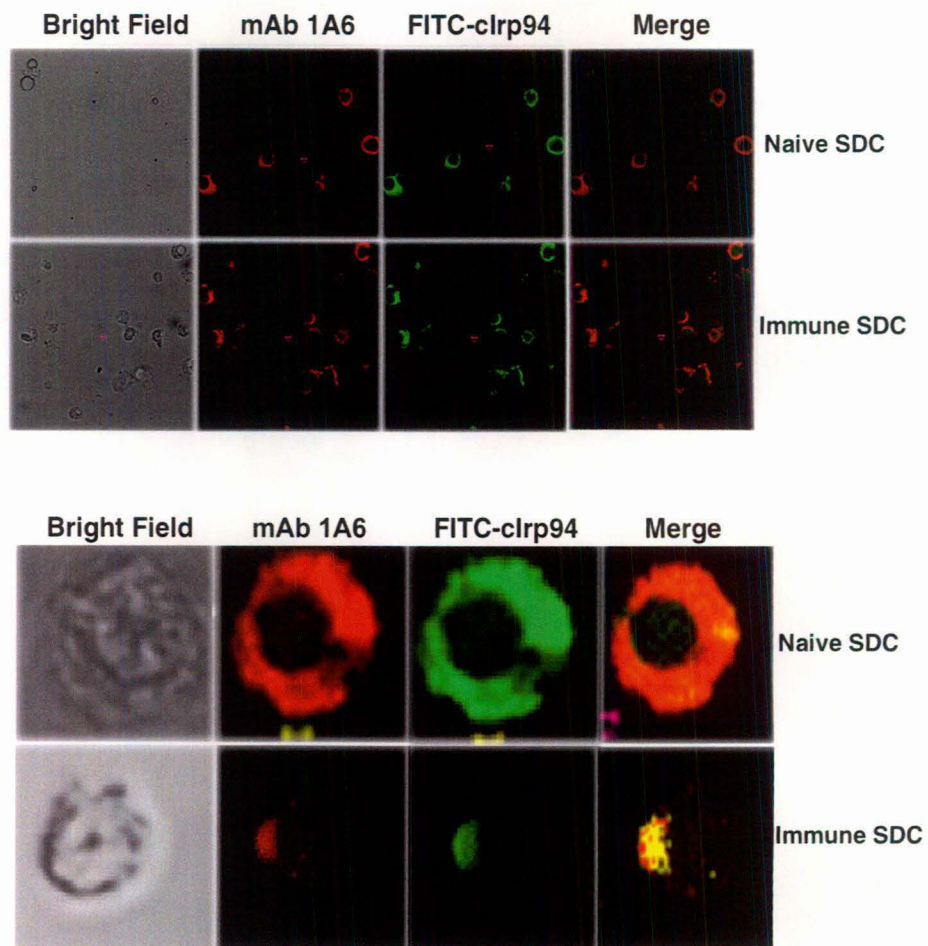
Figure 4.13

Confocal scanning of NKR-P2 distribution on naïve (from Normal rats: Upper panel) and immune (from AK-5 s.c tumor bearing rat: Lower panel) SDCs(red)and corresponding binding of FITC-cIrp94 (green) to NKR-P2 (red). FITC-cIrp94 and NKR-P2 colocalizes all along the cell periphery on naïve SDCs in contrast to clustered colocalization on immune SDCs. Merge image (yellow) corroborate the binding of FITC-cIrp94 to NKR-P2. Upper panel (40X) shows multiple cells in field and lower panel shows a single cell scan in the field at higher magnification. (100X). NKR-P2 staining was performed with mAb 1A6 followed by staining with secondary Ab alexa-594 (red). Transmission images of corresponding DCs are also shown in left panel for both upper and lower panels.

A
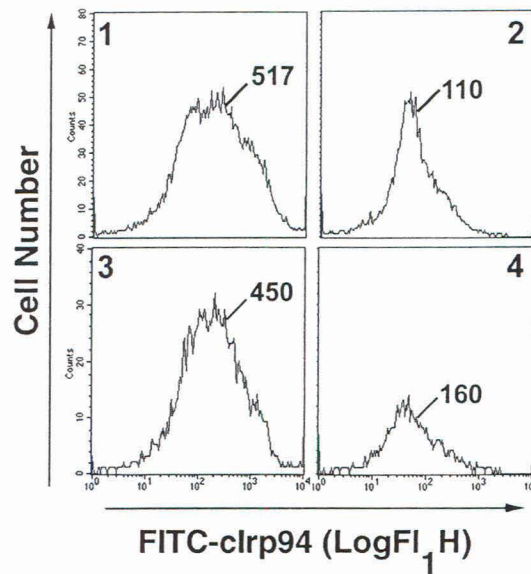
B
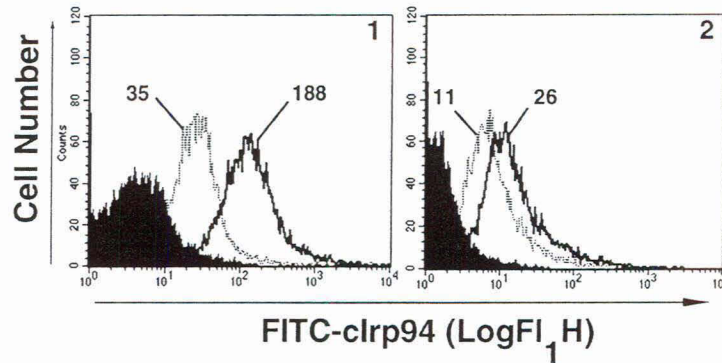
Figure: 4.14

A. Competitive Binding of FITC-cIrp94 to BMDCs: Panels 1 and 3 represent positive controls of FITC-cIrp94 stained BMDCs. Panel 2, represents cells pretreated with mAb 1A6 (anti-NKR-P2 mAb) to block NKR-P2 receptor followed by staining with FITC-cIrp94. Similarly, Panel 4 shows cells pre-blocked with unlabeled recombinant cIrp94 before staining with FITC-cIrp94. In both the cases significant reduction in FITC-cIrp94 binding was observed. (Numbers in figure denotes respective MFI of histogram.)

B. Specific binding of FITC-cIrp94 to NK cells and rat resting CD8$^+$ T cells: Purified NK cells (1) (NK1.1 positive), and T (2) (CD3 positive) cells were stained with FITC-cIrp94 (open histogram–bold line) or pre-blocked with mAb 1A6 and stained with FITC-cIrp94 (open histogram – dotted line). Binding intensity (MFI) is represented in numbers along with histogram. Filled histogram denotes autofluorescence of NK (left panel) and T cells (right panel). One representative experiment of two experiments is shown
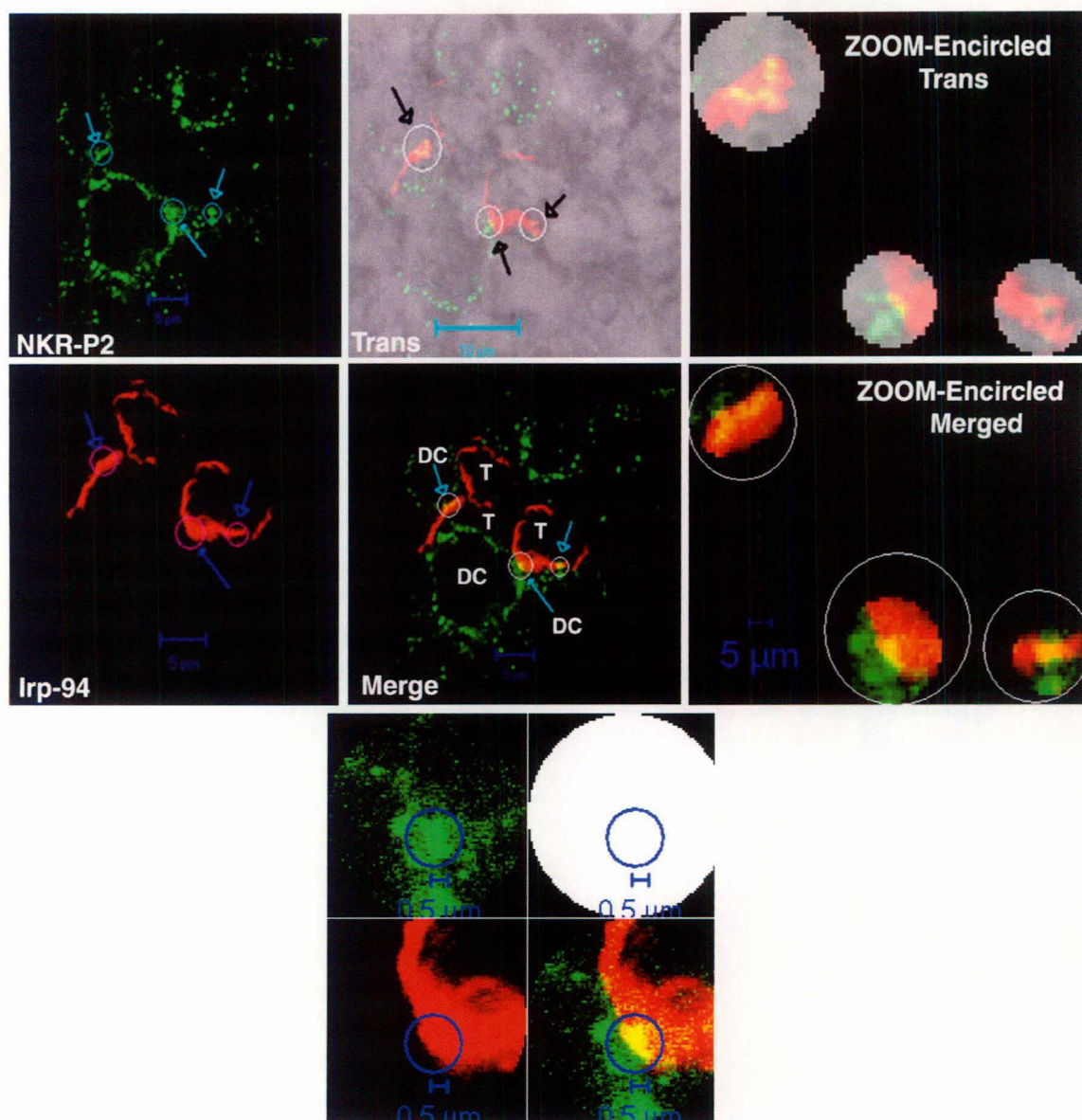
Figure 4.15

Interaction of Irp94 and NKR-P2 at surface in AK-5/DCs conjugate staining: Live AK-5 cells were stained with 2F4 mAb followed by alexa-594 to obtain red Irp94 at AK-5 cells surface; live splenic DCs were stained with mAb 1A6 and alexa-488 to obtain green NKR-P2 at surface. Live and stained AK-5 and DCs together were centrifuged at 1000 rpm for 20min and pellet was further incubated for 30 min at 4^0C to make conjugates. Pellet was gently plated on poly-L-lysine coated coverslip for adherence, washed and scanned on confocal microscope.

At contact points (arrow heads) between DCs and AK-5 tumor cells, colocalization (yellow) were found, which depicts direct recognition of Irp94 with NKR-P2 on cell surface. Zoomed images are also shown in right panels. Lower panel show the interaction (encircled area) at high resolution of one contact point.

manner as a response to cIrp94-NKR-P2 interaction. The production of NO induced by NKR-P2 ligation or LPS stimulation was significantly inhibited by reversible inhibitor L-NAME (200μM), which acts as a competitive substrate of iNOS **(Fig. 4.16A)**. Time dependent NO secretion was measured in response to cIrp94 interaction and significant increase was seen at 24 h **(Fig. 4.16B)**. However, NO production was similar to unstimulated BMDCs with BSA at similar protein concentrations.

iNOS status in cIrp94 stimulated BMDCs was also checked by looking at iNOS transcript levels by RT-PCR **(Fig. 4.17A)** and iNOS protein by immunoblotting **(Fig. 4.17B)** and FACS **(Fig. 4.18)**. The iNOS mRNA and protein levels were increased significantly upon cIrp94 interaction.

In Irp94, interacting domain resides at the C-terminal segment, the N-terminus protein that possess only ATPase domain failed to elicit NO production from BMDCs. However, both C-terminal (~60 kDa) and full length (94 kDa) Irp94 activated BMDCs to release NO **(Fig. 4.19)**. Differential response of non-interacting (nIrp94) and interacting domain (cIrp94 or FlIrp94) also demonstrates the interaction to be specific and through the c-terminal subdomain of Irp94. To ensure that cIrp94 mediated DCs activation is through NKR-P2 on DCs, we pre-block NKR-P2 with commercially available anti-NKG2D polyclonal Ab and found a significant decrease in NO production confirming the response of cIrp94 through NKR-P2 on DCs **(Fig.4.20)**.

4.2.7 Irp94 mediated DCs activation is devoid of LPS contamination

To exclude endotoxin contamination as a cause of responsiveness to cIrp94 and other proteins, we performed *Limulus amebocyte lysate* (Biowhittakar) assay and the detectable endotoxin content was found to be <0.006U/mg of Irp94 preparations, which is within the sensitivity limits of the assay (0.125EU). Polymyxin B is a peptide antibiotic, which neutralizes LPS action by chemical coupling to Lipid A (Storm et al., 1977). Heat denaturation abolished the cIrp94 activating ability of BMDCs, whereas it had negligible effect on Polymyxin B neutralization, which was used to ensure inhibition of lipid-A mediated action of LPS. In parallel assays, LPS activity was completely abolished with polymyxin-B neutralization, whereas LPS activity was consistent upon heat denaturation **(Fig. 4.21)**. These observations, when put together, completely rule out the possibility of LPS contamination in our experimental reagents.

A
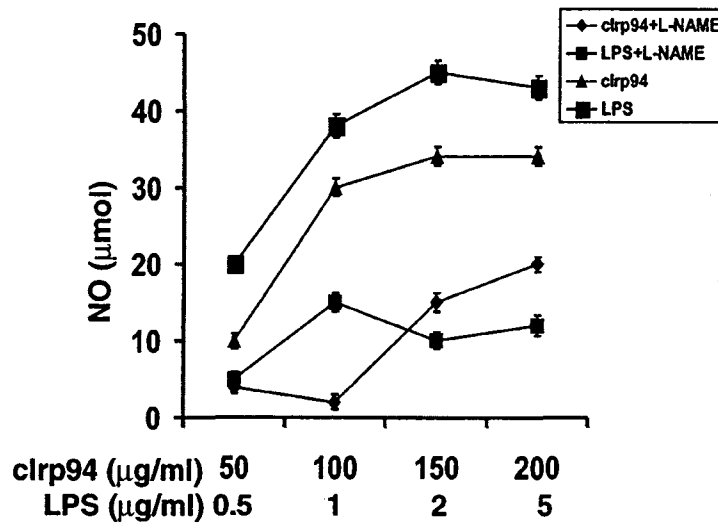
B
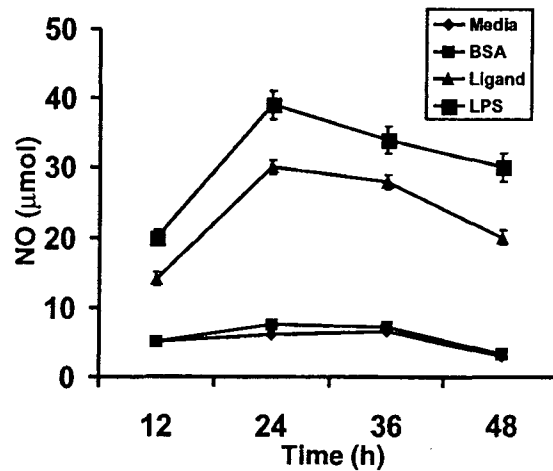
Figure 4.16

A. Dose dependent production of NO with cIrp94 (50-200 μg/ml) and LPS (0.5-5μg/ml). Production of NO was significantly reduced in presence of L-NAME (200μM; competitive substrate of iNOS) for both cIrp94 and LPS. LPS mediated activation and its inhibition with L-NAME was used as experimental positive control. NO content was measured at 24h after stimulation using Griess reagent.

B: BMDCs were incubated with 100 μg/ml of BSA, cIrp94 (Ligand) and 1μg/ml LPS for different time periods and NO content was measured by Griess reagent. Maximum NO production was observed at 24 h of incubation. BSA had minimal effect on NO production.

A

B
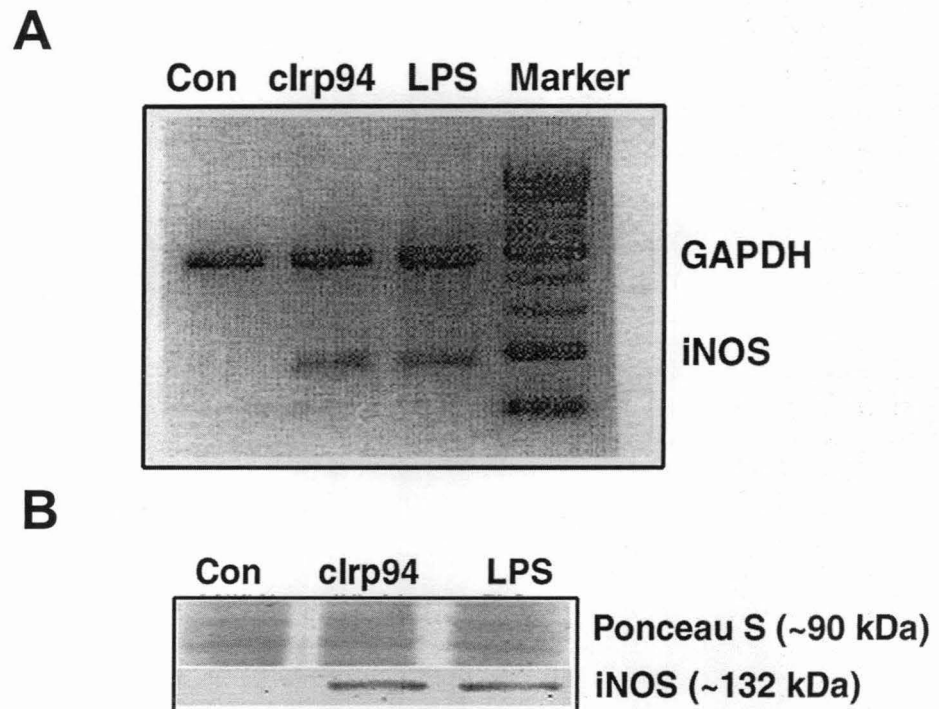
Figure 4.17

A. RT-PCR analysis of iNOS transcript upon treatment with cIrp94 or LPS. Upregulation of iNOS transcript was observed with cIrp94 and LPS stimulated DCs. GAPDH band represents loading control. (Amplification of GAPDH and NKR-P2 was performed in same reaction tube). PCR product size ~ 200bp depicts iNOS and band at ~500bp depicts GAPDH amplification that was used to ascertain equal loading of cDNA template. (Marker: 100bp).

B: cIrp94 induced expression of iNOS protein in BMDCs after 24 h treatment with either cIrp94 or LPS. iNOS protein levels were determined by immunoblotting using anti-mouse iNOS mAb.(Ponceaus S stained blot is also shown to depict equal loading of protein sample).
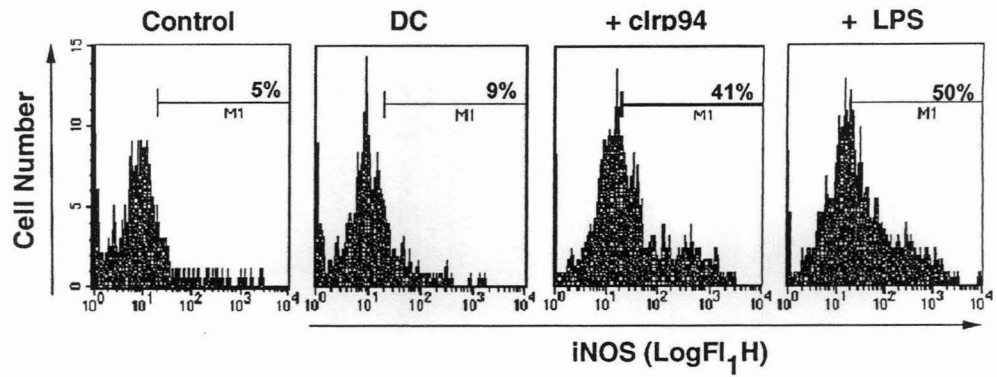
Figure 4.18

Intracellular iNOS protein levels were determined after 24 h treatment with cIrp94 or LPS on day 6 BMDCs, followed by analysis on FACS. Methanol permeabilisation of BMDCs was performed before probing with iNOS specific Ab.
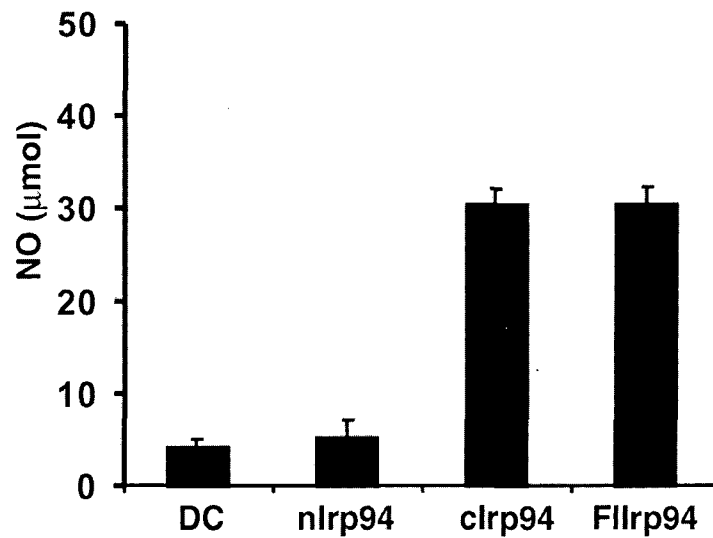
Figure 4.19

NO production with different domains (deletion constructs) of Irp94 (100 µg/ml): nIrp94, cIrp94 and FlIrp94 recombinant proteins (100 µg/ml each) were incubated with day 6 BMDCs, and the NO content was measured after 24 h. nIrp94 protein failed to activate BMDCs, whereas significant release of NO was found with cIrp94 and FlIrp94. Results shown are representative of three similar experiments.
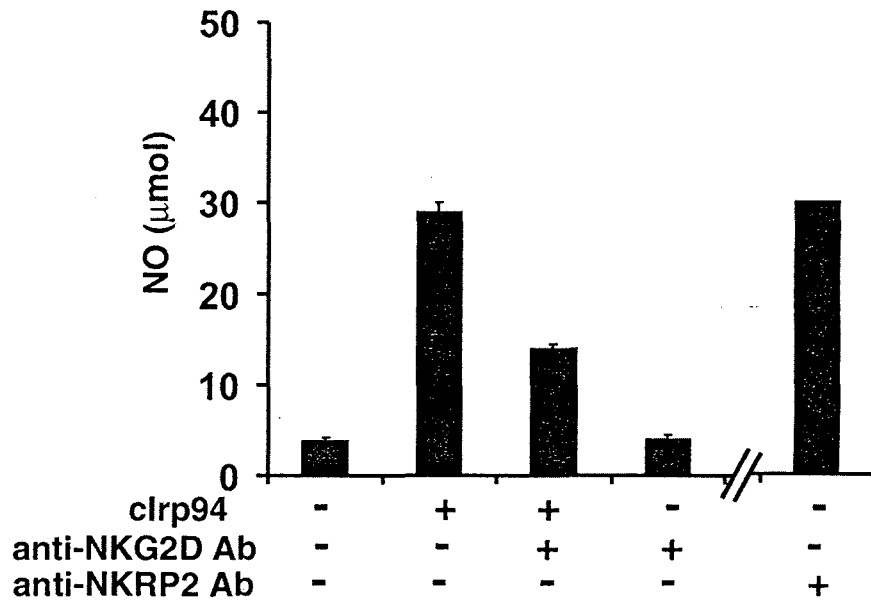
Figure 4.20

Irp94 activates BMDCs through NKG2D/NKR-P2: Control BMDCs or NKR-P2/NKG2D pre-blocked BMDCs (with anti-NKG2D polyclonal commercial Ab) were incubated with cIrp94 for 24 h along with relevant control and NO content was measured in the culture supernatant. Stimulatory action (agonistic nature) of anti-NKR-P2 mAb1A6 is also shown in splitted bar that could not be used in this blocking experiment. The data is representative of two experiments.
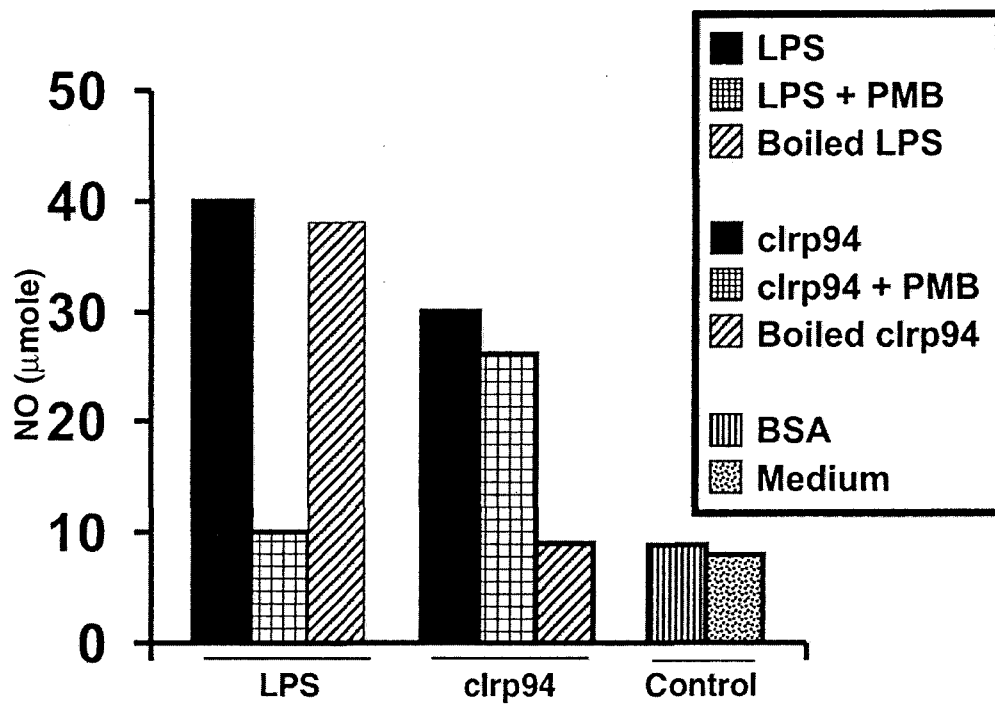
Figure 4.21

The affect of recombinant cIrp94 is not associated with LPS contaminations. NO production of BMDC stimulated with cIrp94. BMDCs were stimulated with cIrp94, BSA and LPS as distinct DC stimulators. Native recombinant cIrp94, cIrp94 in presence of 50 μg/ml PMB and heat-denatured cIrp94 (100°C, 10 min) were three separate forms of Irp94. Heat denatured (boiled) cIrp94 failed to activate BMDCs whereas PMB-coupled cIrp94 retained functional properties of cIrp94, In the parallel assay heat inactivation did not inhibit LPS activity, which was significantly brought down with PMB neutralized LPS. BSA treatment for 6 h did not show any activation and was used as a negative control. Representative bar diagram shown is indicative of more than 10 separate experiments, with different batches of Ni-NTA column purified cIrp94 throughout this study.

4.2.8 Role of NO in NKR-P2 mediated apoptosis of tumor cells

Our earlier observations on DCs activation with agonistic anti-NKR-P2 mAb 1A6 and with fixed AK-5 cells (Alli et al., 2004) prompted us to evaluate Irp94 induced cytotoxic action of BMDCs against tumor. iBMDCs induced apoptosis in BC-8 tumor targets when cocultured for 24 h. Pre-treatment of iBMDCs with cIrp94 enhanced killing of tumor cells, which strengthens our earlier observation that NKR-P2 acts as an activation receptor and Irp94 is its ligand present on the tumor cell surface, which mediates a basal level of tumor cell killing even with uninduced BMDCs.

We have measured induction of apoptosis in BC8 cells under different conditions. cIrp 94 treatment of BMDCs caused 3-fold increase in apoptosis, while cIrp94 treatment in conjunction with W1400 (iNOS specific inhibitor, 5μM), EGTA (Ca^{2+} chelator, 2mM) resulted in significant reduction of apoptosis. Preneutralisation of cIrp94 with mAb 2F4 also resulted in inhibition of apoptosis **(Fig. 4.22)**. Whereas, mAb 2F4 itself did not induce apoptosis in BC8 cells. A parallel and comparable effect of these inhibitors viz. W1400, EGTA and mAb 2F4 was visible as an inhibition of NO release in case of cIrp94 activated BMDCs **(Fig.4.23)**. These observations are highly suggestive of the fact that Ca^{++} mobilization modulates NO secretion upon NKR-P2-Irp94 interaction and as a consequence regulates effector function of BMDCs.

4.2.9 Irp94 ligated DCs augment NK cell cytotoxicity through IL-12

A significant increase (5 fold) in IL-12p40 levels was detected in the supernatants of cIrp94 and FIIrp94 stimulated BMDCs, whereas BMDCs treated with nIrp94 did not show increase in IL-12p40 production, thereby supporting our conclusions regarding the nonfunctional nature of nIrp94 **(Fig.4.24A)**. In order to check whether IL-12 produced by activated BMDCs could augment cytotoxic potential of NK cells, we performed 4 h ^{51}Cr-release assay with NK cells cocultured with cIrp94 pulsed DCs against YAC-1 cells at 20:1 ratio. In the presence of cIrp94 activated DCs, NK cell cytotoxicity against YAC-1 showed 4-fold increase, which was inhibited by neutralizing with anti-IL-12 mAb. However, neither untreated nor cIrp94 induced BMDCs showed any significant cytotoxicity against YAC-1 in 4h ^{51}Cr release assay **(Fig. 4.24B)**.
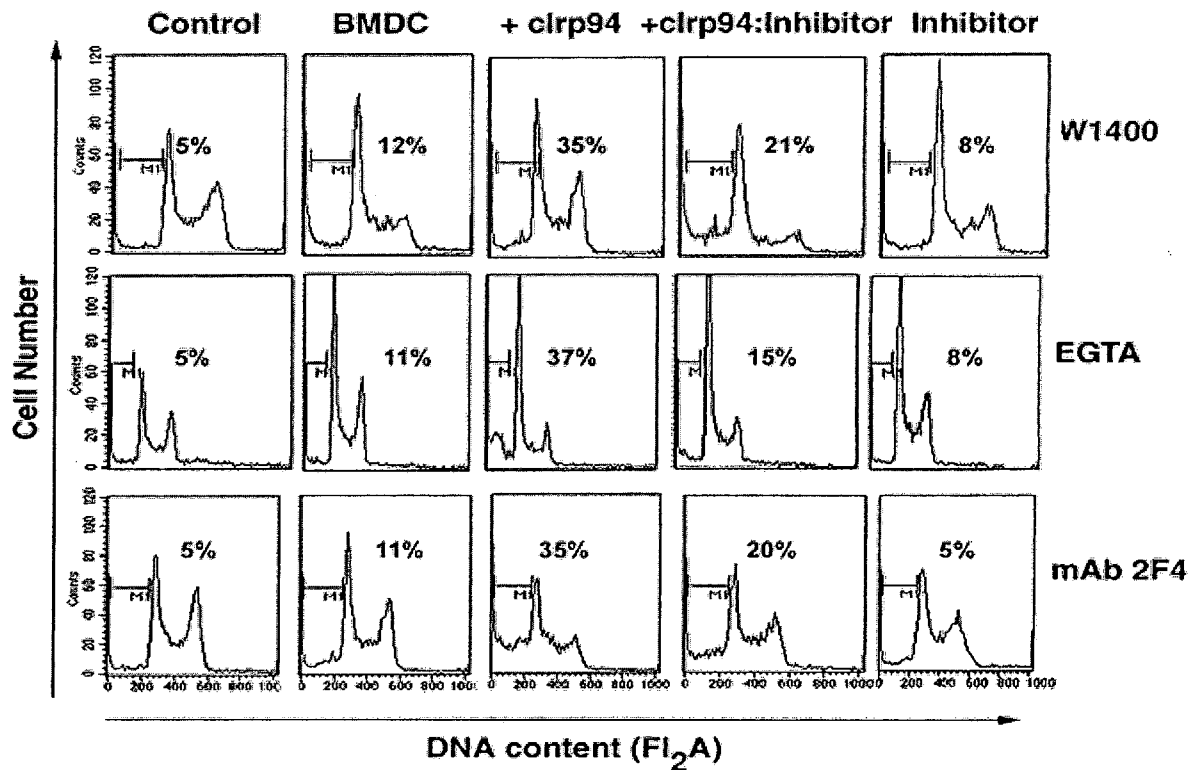
Figure 4.22

Firmly attached BMDCs were activated with cIrp94 for 6 h and cocultured with nonadharent BC-8 cells at 5:1 ratio (E: T) for 24 h. In parallel experiment, BMDCs were pre-treated with W1400 ($2\mu M$/1h), EGTA ($200\mu M$/30min), or mAb 2F4 pre-neutralised cIrp94 (mAb2F4 $200\mu g$/2h/4^0C) and co-cultured with BC-8 tumor targets. Nonadherent BC-8 tumor cells were harvested carefully. Cell pellets were fixed in 80% methanol and percentage apoptotic cells (hypodiploid BC-8 tumor cells) were scored by flow cytometry after staining with propidium iodide.
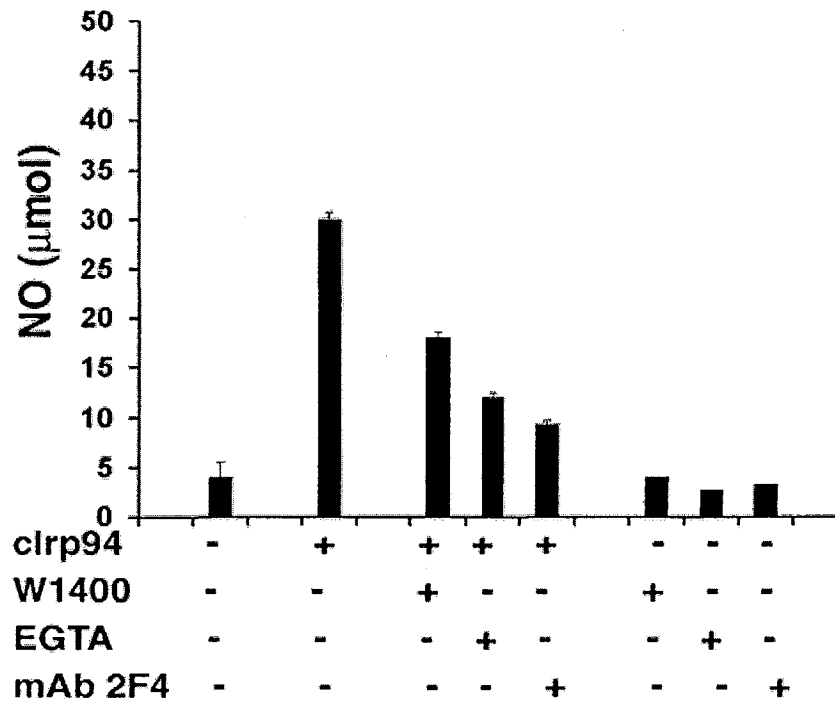
Figure 4.23

Attached BMDCs were stimulated with cIrp94 in conjunction with W1400 (2μM/1h), EGTA (200μM/30min), mAb 2F4 neutralised cIrp94 mAb 2F4 (200μg/2h/4^{0}C) and NO content was estimated in the cell free culture supernatants after 24 h,with Griess reagent.

A

B
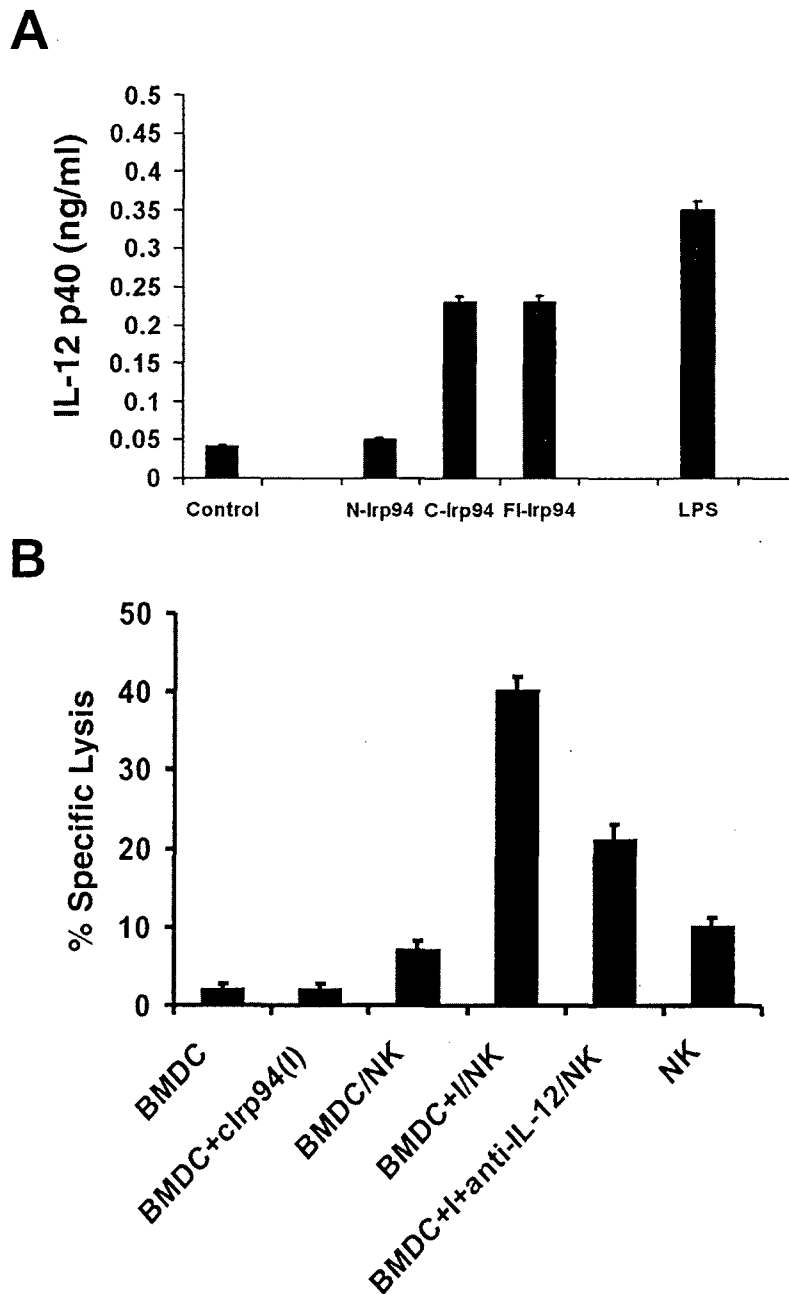
Figure 4.24

A. BMDCs were stimulated with nIrp94, cIrp94, FlIrp94 and LPS. After 24h of stimulation IL-12 levels in the control, nIrp94, cIrp94, FlIrp94 and LPS treated BMDC culture supernatants were estimated by ELIFA.

B. Role of IL-12 secreted by cIrp94 stimulated BMDC on NK cell activation. IL-12 produced by cIrp94 activated BMDCs enhanced the cytotoxicity of NK cells and was neutralized by anti-IL12 mAb, which was measured by NK cell cytotoxicity against YAC-1 in 4h Cr^{51} release assay. No YAC-1 killing was observed with BMDCs directly, which was used as negative control is the assay.

4.2.10   Irp94 ligation of NKR-P2 drives DCs maturation

Effect of cIrp94 on the expression of BMDCs maturation markers like MHCII, B7-2 and CD1a was examined, which are important for Ag-presentation by DCs. After 24 h induction with cIrp94, the surface expression of maturation markers increased significantly in comparison to LPS, which was used as a positive control (Fig. 4.25). The functional significance of Irp94 mediated DCs maturation was assayed in MLR assay. When compared with medium and nIrp94 as control, BMDCs pulsed with cIrp94 and FlIrp94 for 3days induced significant autologous T-cell proliferation (Fig. 4.26A). The stimulatory capacity of cIrp94 was significantly blocked by pre-neutralisation with anti-Irp94mAb 2F4 (Fig. 4.26B).

4.2.11   Irp-94/NKR-P2 mediated translocation of NF-κB to nucleus and regulation of signaling

To investigate the signaling mechanism triggered by cIrp94 ligation on BMDCs, we used several inhibitors of signaling cascade. BMDCs were pretreated with the inhibitors prior to cIrp94 treatment and the resulting effect on BMDCs was read out as their NO secreting capacity. The results obtained show involvement of PI3K (Wortamannin, Ly294002), ERK (PD98059), serine/ threonine phosphatase (okadaic acid), protein kinase C (H7) and protein tyrosine kinase (Genistein) as the specific mediators of cIrp94 mediated BMDCs activation (Fig. 4.27A). NF-κB inhibitory peptide SN50 significantly reduced the Irp94 induced NO production by BMDCs, which suggests the involvement of NF-κBp50 subunit in the stimulation (Fig. 4.27B). NF-κB is activated in the early stages of iNOS induction and Ags presentation function of DCs. iNOS being a well-known target of NF-κB prompted us to check the activation status of NF-κB upon cIrp94-NKR-P2 interaction. The nuclear factor p65 molecule translocated to the nucleus within 2 h after activation of BMDCs with cIrp94 and LPS, as recorded by confocal microscopy (Fig. 4.28A). Degradation of IκB-α was also observed in a time dependent manner with cIrp94 stimulation, thereby confirming the involvement of NF-κB in Irp94-NKR-P2 interaction dependent BMDCs activation (Fig.4.28 B).

4.2.12   Surface Irp94 expression correlates with tumor immunogenicity

Recent studies have demonstrated a strong correlation between tumor immunogenecity and hsp expression. In a dose dependent manner s.c. transplantation of AK-
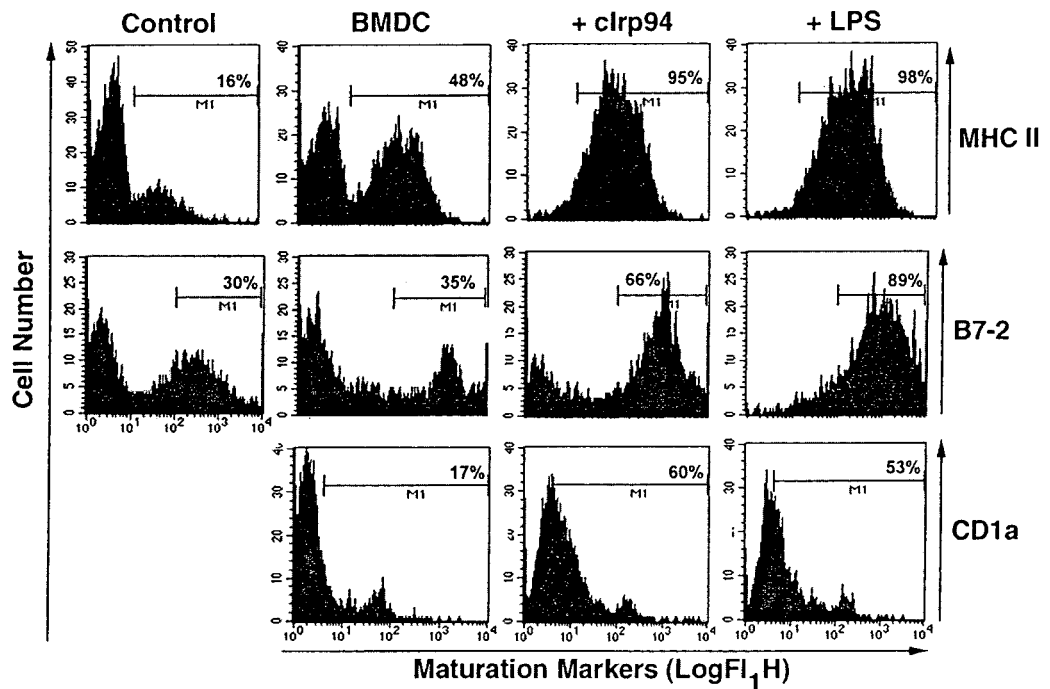
Figure 4.25

Immature BMDCs were stimulated with cIrp94 (100 µg/ml) and LPS (1µg/ml) for 24h. Afterward, DCs maturation markers, MHC II, B7-2 and CD1a was analyzed by using specific mAbs. MHC II, B7-2 expression was determined by appropriate primary mAbs followed by staining with secondary Ab (alexa-488). Mouse alexa-488, and rat alexa-488 was used for the detection of MHC II and B7-2 expression respectively and shown in left panel as control. A FITC-tagged CD1a specific mAb was used to stain DCs. %-positive cells were analyzed by flow cytometry and are shown under the marking M1.

A

B
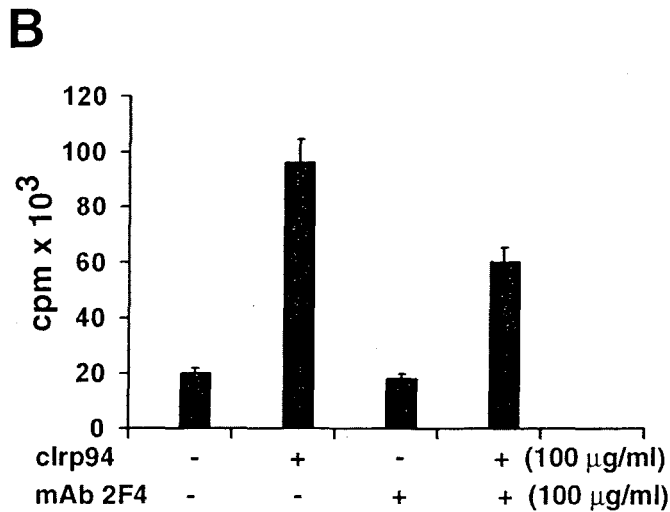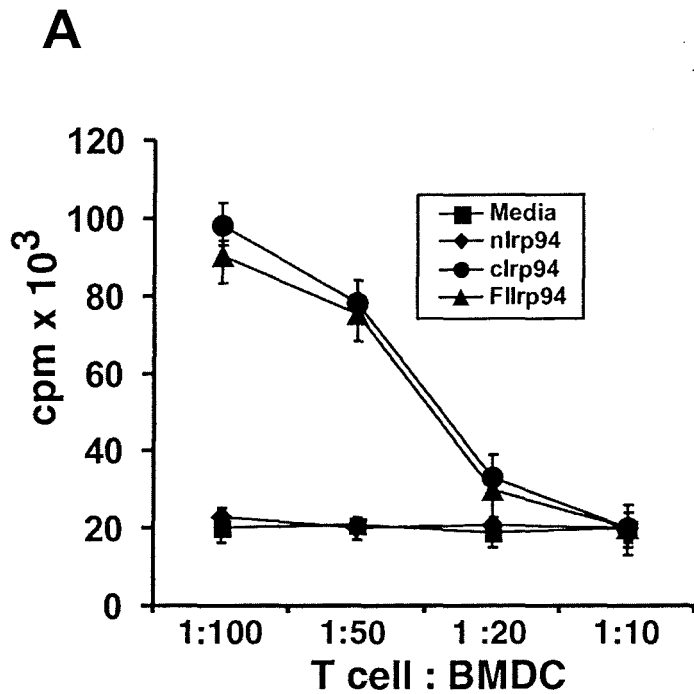
Figure 4.26

A. DCs were incubated with different fragments (products of deletion constructs) of Irp94 for 24 h. Cells were incubated with autologous T-cells at different (T: DCs) ratio for 3days in conjunction with Irp94 fractions. 18 h before T cells harvesting H^3-thymidine (1μCi/well) was added. Cells were harvested, washed and the incorporated radioactivity ([H]^3-thymidine) was counted along with enhancer cocktail. Represented data depicts the proliferation index of stimulated T-cells in counts per minutes (cpm).

B: cIrp94 and pre-neutralized cIrp94 (cIrp94: mAb 2F4) were used as DCs stimulators in MLR assay as described above and T cell proliferation was measured. The data shown is representative of 3 similar experiments.

A

Control
clrp94

NO (μmol)

clrp94　Worta.　Ly294002　PD98059　O.acid　H7　Genistein

B

NO (μmol)

Irp94 (100 μg/ml)	—	+	+	+
SN50 (50 μg/ml)	—	—	+	—
SN50M (50 μg/ml)	—	—	—	+
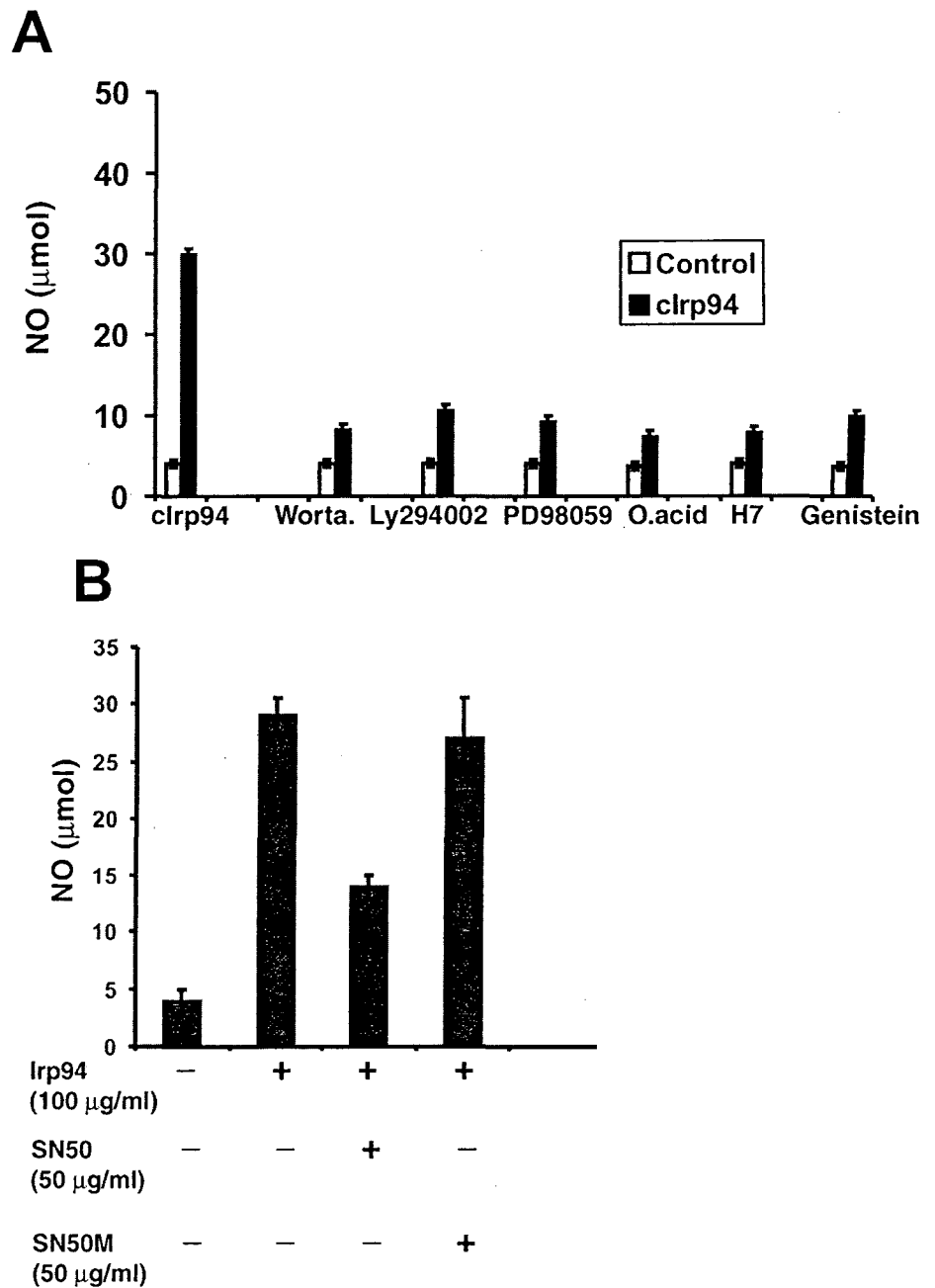
Figure 4.27

A. The affect of protein kinase and phosphatase inhibitors on NO production by cIrp94 treated BMDCs was estimated. BMDCs were incubated with $2\mu M$ wortammanin, $20\mu M$ Ly294002, $25\mu M$ PD98059, $10\mu M$ okadaic acid, $10\mu M$ H7, $10\mu M$ genestein in presence or absence of $100\mu g/ml$ cIrp94 for 24h.

B. Inhibition of Irp94 induced NO production by SN50 (NF-κB-inhibitory peptide):BMDCs were preincubated with SN50 (Active) and SN50M (Inactive control peptide) for 2h as described by the supplier, followed by treatment with cIrp94 for 24h.

NO content was measured in culture supernatants of control and inhibitor treated DCs using Griess reagent.

A

BMDC	+ cIrp94	+ LPS	
			NFκB
			Nucleus
			Merge
			Bright Field

B

Control   30   60   90   120   (Time min)

IκB (48 kDa)

Ponceau S (~60 kDa)
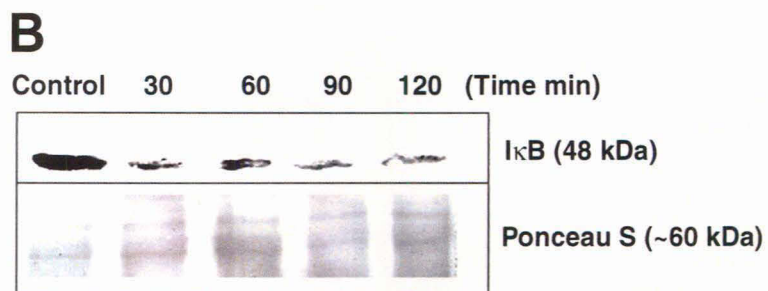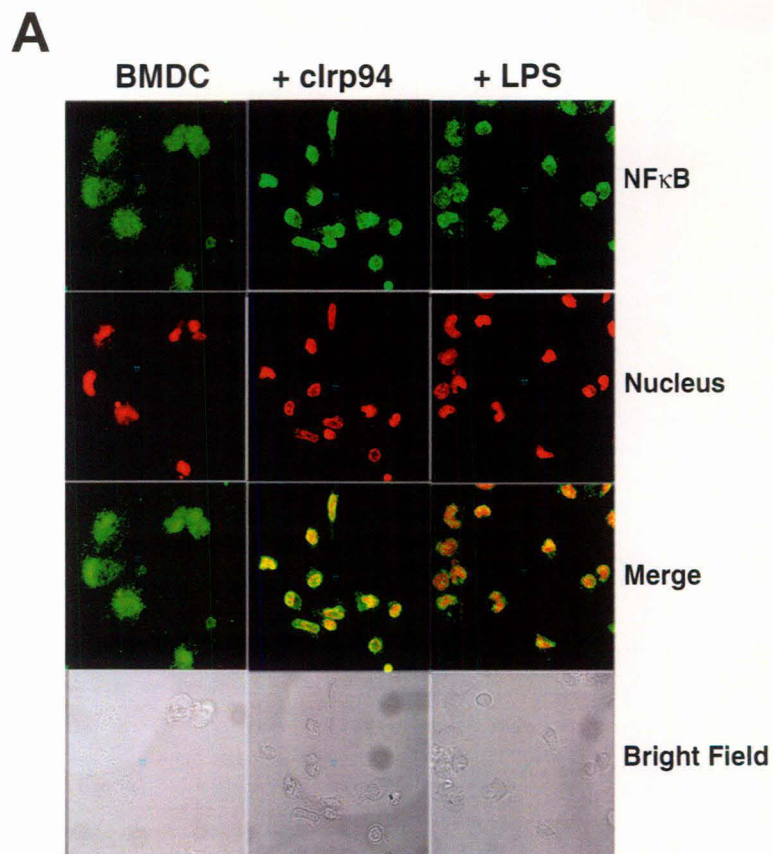
Figure 4. 28

A. cIrp94 induced translocation of NF-κB to the nucleus. BMDCs were induced with cIrp94 on coverslip for 2 h, fixed and permeabilised with methanol:acetone (1:1) for 30 min. Washed cells were then probed with rabbit anti-NF-κB polyclonal Ab and stained with FITC-conjugated secondary Ab (alexa-488:green). Nucleus was stained with propidium iodide (red) before confocal scanning. Yellow (Merge) colour is evident in the nucleus in cIrp94-stimulated cells suggesting translocation of NF-κB to the nucleus; LPS induced NF-κB translocation was used as positive control (60X). Transmission images of cells are also shown.

B. Degradation of IκB in BMDCs treated with cIrp94: (10^6cells/well) BMDCs were induced with cIrp94 for different time periods as indicated. Cells were lysed and immunoblotted with rabbit anti-mouse IκB Ab. Ponceau-S blot is also shown for equal loading of samples.

5 cells leads to solid tumor formation. We have found sustained surface expression of Irp94 during solid tumor growth, which regresses spontaneously, whereas surface expression of Irp94 sharply declines during AK-5 ascites progression (i.p.), which leads to the death of the host **(Fig. 4.29)**. Similar decline pattern in surface Irp94 was also observed in ZAH ascites **(Fig. 4.30A)** and MethA ascites **(Fig.4.30B)** that lead to the death of the hosts. Sustained surface expression of Irp94 in s.c. tumor retains the competence to induce DCs **(Fig.4.31A)** whereas decreased stimulatory potential was observed by ascitic tumor **(Fig.4.31B)**, suggesting the implications of surface Irp94 expression in immune responses *in-vivo*.

4.3 DISCUSSION

Various c-type lectin like receptors (CLRs) participate in self and non-self recognition by DCs (Geijtenbeek et al., 2004). Fas-FasL interaction, TRAIL upregulation and tumoricidal action of DCs have been demonstrated earlier with native and with various subsets of DCs (Lu et al., 2002; Schmitz et al., 2005; Shi et al., 2005). In this study we have shown a novel surface interaction between rat BMDCs and tumor cells (NKR-P2-Irp94), which mediates specialized killing of tumor, and corroborated the direct susceptibility of tumor cells.

NKG2D-Ligand system has been assessed as a multifunctional skill to activate innate immune system in response to tumor and virus infected cells. NKG2D ligand non-redundancy has been observed in intraspecies conditions, such as MICA/B that was initially identified for human NKG2D, has striking dissimilarity with mouse Rae1β, H60 and Mult1. Among themselves Rae1β, H60 and Mult1 show only ~ 30% similarities in amino acids sequence but are completely different from MICA/B. However, human UL16 binding protein (ULBPs), which was identified using a different approach, has very little similarity with mouse NKG2D ligands (Raulet, 2003). Very recently a ULBPs homolog (RAET1E) has been found that does not contain transmembrane domain but interacts with NKG2D extracellularly upon secretion (Cao et al., 2007). A recent report has also demonstrated interaction between MICA (human NKG2D ligand) with HSPs (hspa5, grp78) in the plasmamembrane of tumor cells (Kaiser et al., 2007).

NKG2D/NKR-P2 is a unique member of c-type lectin like receptor family and binds to its ligands with variable affinities by dimeric surface. The dimeric surface is rich in basic amino acids and contributes to the recognition of dissimilar ligands. However overall shape complementarity is an imperative factor for ligand recognition (Radaev et al., 2002). Thus, NKG2D-ligand system works in a broad range of diversity but mounts an efficient immune
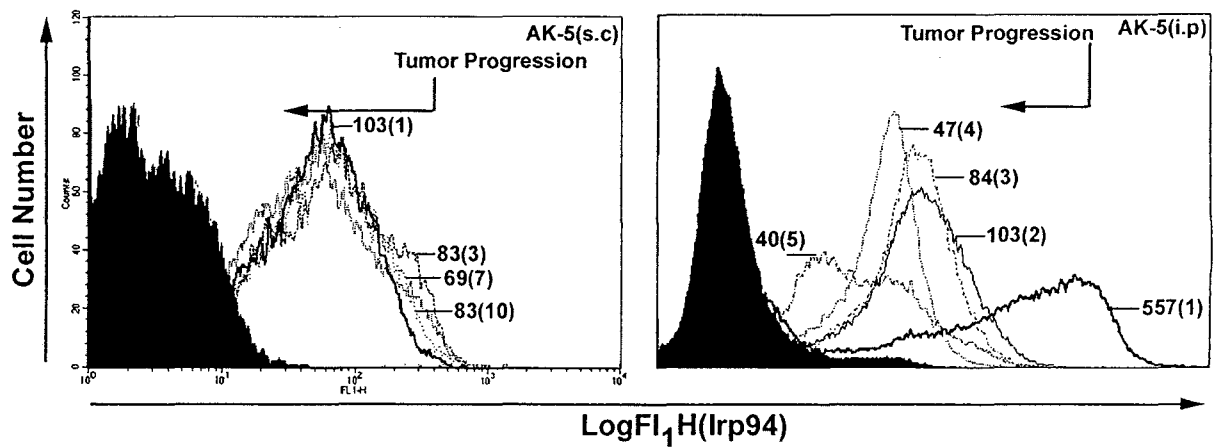
Figure 4.29

20x10^{6} AK-5 (histiocytoma) tumor cells were transplanted s.c. in wistar rats (grow as solid tumor before regression) and i.p. (grow as ascites leading to the death of host). AK-5 cells were collected from s.c and i.p tumor bearing rats on indicated days and plated for 2 h for the removal of resident macrophages. Live non-adherent AK-5 cells were stained with anti-Irp94 mAb followed by secondary Ab (alexa-488). The level of surface Irp94 was determined by FACS and demonstrated as MFI in numbers (Filled histogram denotes secondary Ab control; open histogram- surface Irp94 (mAb 2F4). Left panel shows staining of surface Irp94 on s.c tumor cells and right panel shows staining of surface Irp94 on ascitic tumor cells.
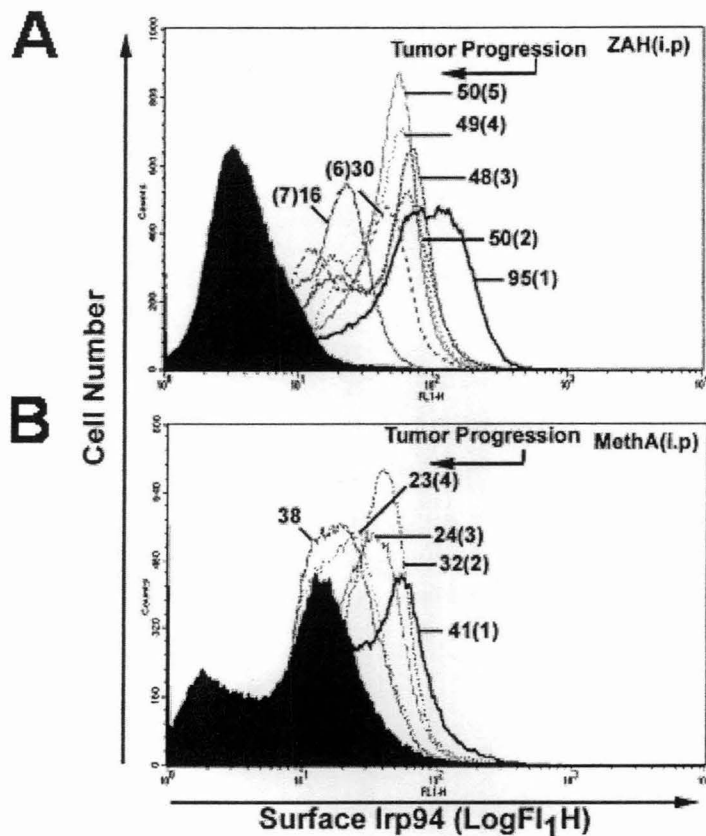
Figure 4.30

A. 20 x 10^6 ZAH (hepatoma) tumor cells were transplanted i.p in wistar rats (leading to ascites development and host death) ZAH cells were collected on indicated days and plated for 2h in complete media for the removal of resident macrophages. Live non-adherent ZAH cells were stained with anti-Irp94 mAb followed by secondary Ab (alexa-488). The level of surface Irp94 was determined by FACS and demonstrated as MFI (Filled histogram denotes secondary Ab control; open histogram- surface Irp94 (mAb 2F4).

B. Meth-A(Fibrosarcoma) tumor cells were transplanted i.p in Balb/c mice(ascites development and host death). Meth-A cells were collected on indicated days and plated for 2h in complete media for the removal of resident macrophages. Live non-adherent Meth-A cells were stained with anti-Irp94 mAb followed by secondary Ab (alexa-488). The level of surface Irp94 was determined by FACS and demonstrated as MFI (Filled histogram denotes secondary Ab control; open histogram- surface Irp94 (mAb 2F4).
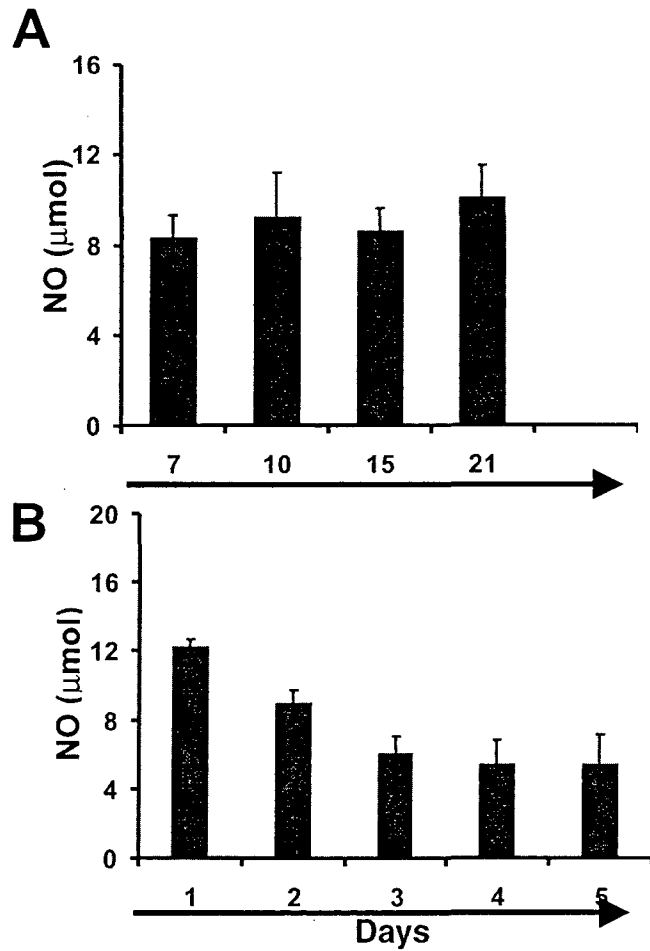
Figure 4.31

A: Different day harvested AK-5 tumor cells (s.c.) were fixed in 1% paraformaldehyde and cocultured with BMDCs (Tumor: BMDC; 25:1 ratio) for 24h. NO content in the culture supernatants was estimated using Griess reagent.

B: Different day harvested AK-5 tumor cells (i.p) were fixed in 1% paraformaldehyde and cocultured with BMDCs (Tumor: BMDC; 25:1 ratio) for 24h. NO content in culture supernatants was estimated using Griess reagent.

response. Our observations about a 110-kDa hsp working as NKR-P2 ligand on rat DCs provides the evidence of additional diversity for efficient tumor recognition system that is also supported by the binding of hsp70 peptide complex to NKG2D. A significant binding of NKG2D to fluorescent-hsp70 peptide complex has been found that contained HSPA1a, hsp70 protein 8, Grp78 and other hsp70 members (Theriault et al., 2006). We have also observed the binding of sNKR-P2 to membrane Hsp70.Cκ transfected CHO cell. Taken together these observations confer a broad range of heat shock protein recognition by NKG2D/NKR-P2. Whereas a wide range of 'MHC I-like' ligands to NKG2D is known (Radaev et al., 2002), however further structural studies are needed to reveal the exact mode of hsp binding to NKG2D/NKR-P2. Heat shock transcription elements regulate expression of hsp, however MICA/B upregulation in many tumors is also thought to be the consequence of activation of heat shock transcription elements in the promoters of corresponding genes; an event known to accompany transformation (Venkataraman et al., 2007). Thus regulation of human NKG2D and rat NKR-P2 ligands with heat shock transcription elements supports a common regulatory system.

Heat shock proteins perform essential functions in the cellular physiology and are usually upregulated under stressed conditions. Presence of multiple splice variants is a common feature of HSPs. HSP70: constitutive and inducible hsp70, hsp72; HSP90: hsp90α, hsp90β. The splice variants of Irp94 (840 AA) is also described that are expressed in human i.e HSP70RY (701 AA) mice i.e APG-2 (841 AA). However the isoforms are not reported to contain or acquire transmembrane region or GPI anchorage properties. Hence it is indeed enigmatic as to how the HSPs are present on tumor cell membrane and precise studies are needed to answer this question. Also, the first report of Irp94 is itself accompanied with presence of isoforms (Yagita et al., 1999). The detection of HSPs (rat Irp94) with 2F4 on tumor cell line of mouse and human origin also confirms the presence of homolog (or splice variants) in other species.

Recently involvement of endolysosome in the transport of Hsp70 to the membrane is demonstrated (Calderwood et al., 2007; Mambula and Calderwood, 2006). Expression of surface gp96 has been found to be evolutionary conserved on various tumors and highly immunogenic tumors express more of gp96 on cell surface (Robert et al., 1999). Hsp72 surface expression on stressed tumor cells also augments cytotoxicity of NK cells (Multhoff, 1997). The recent report provides the evidence of surface expression of hsp90 on B cell lymphoma upon Epstein Barr Virus infection (Kotsiopriftis et al., 2005), expression of hsp60 on stressed endothelial cells (Pfister et al., 2005) and hsp72 expression on neutrophils upon

LPS stimulation (Hirsh et al., 2006), suggesting surface expression of hsps under stressed conditions. Hsps can elicit the immune responses if expressed on plasmamembrane (Multhoff and Hightower, 1996). In transgenic model, enforced surface hsp expression leads to autoimmune disorder (Liu et al., 2003). The surface expression of Irp94 by many tumor cell lines but not by normal cells also suggests that cellular stress induces upregulation of Irp94. These findings support our observations regarding surface expression of Irp94 on tumor cell, which increases upon heat shock on AK-5 cells. During necrotic and apoptotic tumor cell death, released exogenous hsps and hsp-immunogenic peptide complexes stimulate APCs through CD91 receptor and initiate anti-tumor immune response (Basu et al., 2001). Similarly, TLR2, TLR4 and CD40 act as receptors for exogenous hsp60 and hsp70 and function as Th1 polarising agents (Ohashi et al., 2000; Asea et al., 2002; Wan et al., 2004). However hsps binding and stimulation through NKG2D/NKRP-2 display a novel mode of action in contrast to distinct pattern recognition receptors. Higher hsps 110 has been shown to act as a "danger signal" to DCs (Manjili et al., 2005), however no specific receptor for these hsps have been assigned yet .Our data suggest that the stimulatory capacity of Irp94 resides in the C-terminal subdomain and the ATPase domain is not necessary for Irp94 recognition by NKR-P2. Similarly, stimulating epitope of microbial hsp70 resides in the base of C-terminus domain and activates DCs through CD40 receptor and adjuvant function of hsp70 on human MoDCs (Wang et al., 2002b). Irp94 contains hsp70 signature, which is broadly conserved for various hsp110 and hsp70 family members (Yagita et al., 1999). We have previously shown AK-5 cells to be deficient in mounting a heat shock response and therefore they undergo apoptosis (Sreedhar et al., 1999). Moreover, AK-5 cells do not express inducible hsp70 on the cell surface. Hence in AK-5, Irp94 functions exclusively as the interacting partner for NKR-P2 receptor.

HSPs are not the usual membrane proteins and their specific structural topology in bilayer membrane has not been investigated or predicted. Irp94 contains hsp70-c-terminal subdomain at 616-699 AA and at 701-794 AA that belongs to HSP70-c-terminal superfamily. The high molecular weight stress proteins hsp110 are related in sequence and structure to the hsp70 family and together are referred to as the "hsp70 superfamily"(Easton et al., 2000; Facciponte et al., 2007; Shaner and Morano, 2007). The extracellular localized element of hsp70 on tumor cells that are accessible to immune cells has been examined and the c-terminal region that contains at least one single ά-helix is defined as recognition element (Botzler et al., 1998b). The hsp70 on tumor cells has been defined as recognition structure for immune cells (Multhoff, 1997; Roigas et al., 1998), additionally a c-terminal resident 14-mer hsp70 peptide

has also been shown to activate immune cells. Functionally, the cell surface localized HSPs also participates in the cytolytic activity of human NK cells (Gastpar et al., 2004), and also enhances the cytotolytic capacity of NK cells (Gross et al., 2003b). The association of surface hsp with CD94 NK receptor has also been shown. These evidences suggest that membrane Irp94 expose the interacting element to mount the immune responses. mAb 2F4 was generated against the c-terminal doamin of Irp94 and stains live tumor cells suggesting the exposure of c-terminal at outer periphery of tumor cells of multiple origin. Recently glucose-regulated protein of 78 kDa, which is a member of hsp70 family, was reported to expose c-terminal domain on the surface of breast carcinomas, lung cancers, and melanomas but not on normal cells. These findings altogether confirm the expression of HSPs on the tumor cells of multiple origins (Jakobsen et al., 2007)

Enhanced binding of FITC-cIrp94 to mature BMDCs and NKR-P2 upregulation with maturation stimuli confirms its specificity to NKR-P2 and supports our earlier observations on NKR-P2 upregulation in activated splenic DCs (Alli et al., 2004), indicating that the mature DCs are more efficient in tumor recognition.

The evidence that cIrp94 is a specific binding partner of NKR-P2 comes from four different sets of observations. First, the GST pull down assay clearly shows the binding of NKR-P2 with Irp94, where Irp94 is pulled down along with ECD of NKR-P2. Secondly, specific binding of sNKR-P2 to ectopically expressed surface Irp94 and binding of FITC-cIrp94 to ectopically expressed NKR-P2. Thirdly, FITC conjugated cIrp94 colocalizes with NKR-P2, displaying a similar expression pattern as NKR-P2 on splenic immune DCs, where both are seen to be concentrated at the same location on the membrane as compared to naive splenic DCs, where colocalisation was observed all along the periphery. Fourthly, in a competitive binding assay, where a marked inhibition of FITC-cIrp94 binding was observed upon pre-blocking of NKR-P2 with mAb1A6 and untagged Irp94 on BMDCs. Similar inhibition of FITC-cIrp94 binding was also observed on NK and T cell upon NKR-P2 preblocking. These independent observations clearly prove the binding specificity of Irp94 for NKR-P2.

We have demonstrated the interaction of Irp94 with NKR-P2 on DCs, which activates them to secrete L-arginine and Ca^{++} dependent NO that in turn induced apoptosis in tumor targets. NO is a well-known tumoricidal molecule that executes tumor cell killing by downregulating cyclin D1, inhibition of vital enzymes essential for tumor growth and by activation of caspases (Bogdan, 2001). In-vitro, iNOS induction in DCs has been shown with Hsps, IFN-γ, endotoxins and upon CD40 ligation (Panjwani et al., 2002 ; Lu et al., 1996).

Enhanced NO production is also reported in mouse thymic DCs in response to self and allo-antigens (Aiello et al., 2000). NO production has also been documented in *in-vivo* conditions (Farias-Eisner et al., 1994; Bhaumik and Khar, 1998; Serbina et al., 2003). Mouse and rat BMDCs have also been shown to perform tumoricidal action through NO upon treatment with LPS or IFN-γ, however no specific surface interaction was defined in these studies (Nicolas et al., 2007; Shimamura et al., 2002). Production of NO by DCs after interaction with ectopically expressed Irp94 on CHO cells again confirms its efficiency during cellular contact, since Irp94-NKR-P2 mediated DCs activation involves interaction of surface proteins and killing by soluble NO. To rule out the susceptibility of DCs during coculture, we performed MTT assay on effector BMDCs, which showed consistent viability of DCs during coculture.

DCs have been shown to produce Th1 polarising cytokine IL-12, in addition to IL-1β; IFN-γ, IL-18, whereas IL-10 and IL-4 production are documented during tolerogenic stages (Gad et al., 2003). We also observed increased IL-12 levels intracellularly and in culture supernatants from cIpr94 activated BMDCs. In AK-5 tumor regression model, IL-12 activated NK cells have been identified as the major effector cells (Khar et al., 1997). Hence we checked whether cIrp94 activated BMDCs could augment cytotoxic action of naive NK cells. We observed DCs mediated activation of NK cells through IL-12 thereby supporting our earlier findings of DCs activation with fixed AK-5 cells *in-vitro* and *in-vivo* (Alli and Khar, 2004) and the anti-tumor cross talk between DCs and NK cells (Degli-Esposti and Smyth, 2005). This crucial activity of Irp94 suggests that it may enhance innate immune response or break immune tolerance status in tumors that regress spontaneously.

In various studies, tumor-infiltrating mDCs have been found to be associated with the regression of the tumor (Byrne and Halliday, 2003). We also observed upregulation of MHC II, B7-2 and CD1a as phenotypic maturation markers upon treatment of DCs with Irp94, which correlates very well with our earlier findings of DCs maturation with fixed AK-5 cells (Alli and Khar, 2004). In autologous MLR assays, Irp94 treated BMDCs exhibit enhanced T-cell proliferative capacity. This phenotypic and functional maturation through NKR-P2 displays crucial adaptive immune response of BMDCs. Earlier, DCs mediated priming of tumor specific T cell immune response has been shown with complex assortments of tumor Ags, however a recent approach of targeting NKG2D ligand (MICA) for generating T cell specific immune response through DCs supports our findings and demonstrates the importance of NKG2D ligand in DCs mediated immunotherapy (Groh et al., 2005). HSPs have been shown to induce NO production and DCs maturation *in-vitro* (Basu and Srivastava,

2003) and recently iNOS has been found to regulate DCs maturation process by inhibiting caspase-like activity of iDCs (Wong et al., 2004). Hence Irp94 mediated iNOS induction and maturation of BMDCs appears to be a concerted action and is assumed to govern both innate and adaptive anti-tumor immune responses.

Since NO is a crucial effector of DCs-mediated cytotoxicity, signal transduction mechanism of Irp94 mediated NO production was investigated. The results from the studies with pharmacological inhibitors demonstrated that NO production with Irp94-NKR-P2 interaction is regulated by PI3 kinase, tyrosine kinase, ERK kinase, serine threonine protein phosphatase, and protein kinase C. These observations suggest the involvement of MAP kinase pathway in NKR-P2 mediated DCs activation. Similar pathways seem to operate in DCs for iNOS induction (Cruz et al., 1999) and with hsp60 interaction in DCs (Flohe et al., 2003). However, it will be interesting to investigate the accurate signal transduction cascade upon NKR-P2 ligation.

NF-κB transcription factor (p50-p65) is the prototype of a family of homodimeric and heterodimeric protein complexes comprised of subunits related to the c-rel protooncogene. NF-κB activation plays an important role in innate and adaptive immunity (Bonizzi and Karin, 2004), and has been found to execute iNOS induction with Hsps (Sauter et al., 2000; Xie et al., 1994). Cytotoxic NO production after Irp94 treatment represents strong induction of iNOS, which is regulated by NF-κB activation. Furthermore, NF-κB acts as a potential transcription factor in the initiation of MHC II, CD86, and CD80 upregulation, as well as in IL-12 and TNF-α production (Yoshimura et al., 2001). We have observed translocation of NF-κB to the nucleus after 2 h treatment with Irp94. cIrp94 treated BMDCs show a strong translocation as compared to LPS in the nucleus, which suggests early activation of NF-κB with NKR-P2 ligation. NF-κB activation takes place due to the phosphorylation and degradation of IκB in cytoplasm (Cruz et al., 1999), data on cIrp94 mediated degradation of IκB-α also supports involvement of NF-κB activation during Irp94-NKR-P2 interaction . Thus our data suggest NF-κB to be a key executioner of Irp94 mediated activation of BMDCs. Earlier role of NF-κB in DCs activation and maturation was reported from exogenously released hsps from necrotic tumor cells. Here, we show the efficiency of surface hsp to induce similar cascade in BMDCs.

Our results demonstrate the role of surface HSPs in eliciting DCs mediated immune responses through NKR-P2/NKG2D. Surface hsp accounts multiple rationales to activate immune cells and also induces activation of DCs (Zheng et al., 2001), and a strong

correlation between hsp expression and tumor immunogenicity is reported (Clark and Menoret, 2001).

Thus, our findings have important implications in host resistance against tumor. Our data highlights novel expression of Irp94 on tumor cell surface and elucidates the mechanism of hsp mediated tumor recognition through DCs. Our data also demonstrate maturation of DCs through the interaction between hsp and NKR-P2, which leads to the generation of adaptive immune response in DCs. Although many cytotoxic subsets of DCs have been described in the literature, our findings suggest a common cytotoxic action of DCs through tumor recognition receptor, since bone marrow precursors generate both lymphoid and myeloid subsets of DCs. Thus NKR-P2 function on DCs establishes a significant link between adaptive and innate immunity through tumor recognition receptor.

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LIST OF PUBLICATIONS

(1) <u>Raghvendra M. Srivastava</u>, B Savithri and Ashok Khar (2003) Activating and inhibitory receptors and their role in natural killer cell function. *Ind. J. of Biochemistry & biophysics* **40**,291-299 (Review)

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(4) <u>Raghvendra M. Srivastava</u> and Ashok Khar. Role of Dendritic cell receptors in antitumor responses. *Current Molecular Medicine.* Invited Review.(Manuscript under preparation)

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