A study on crosstalk between *lung carcinoma* and *immune cells*

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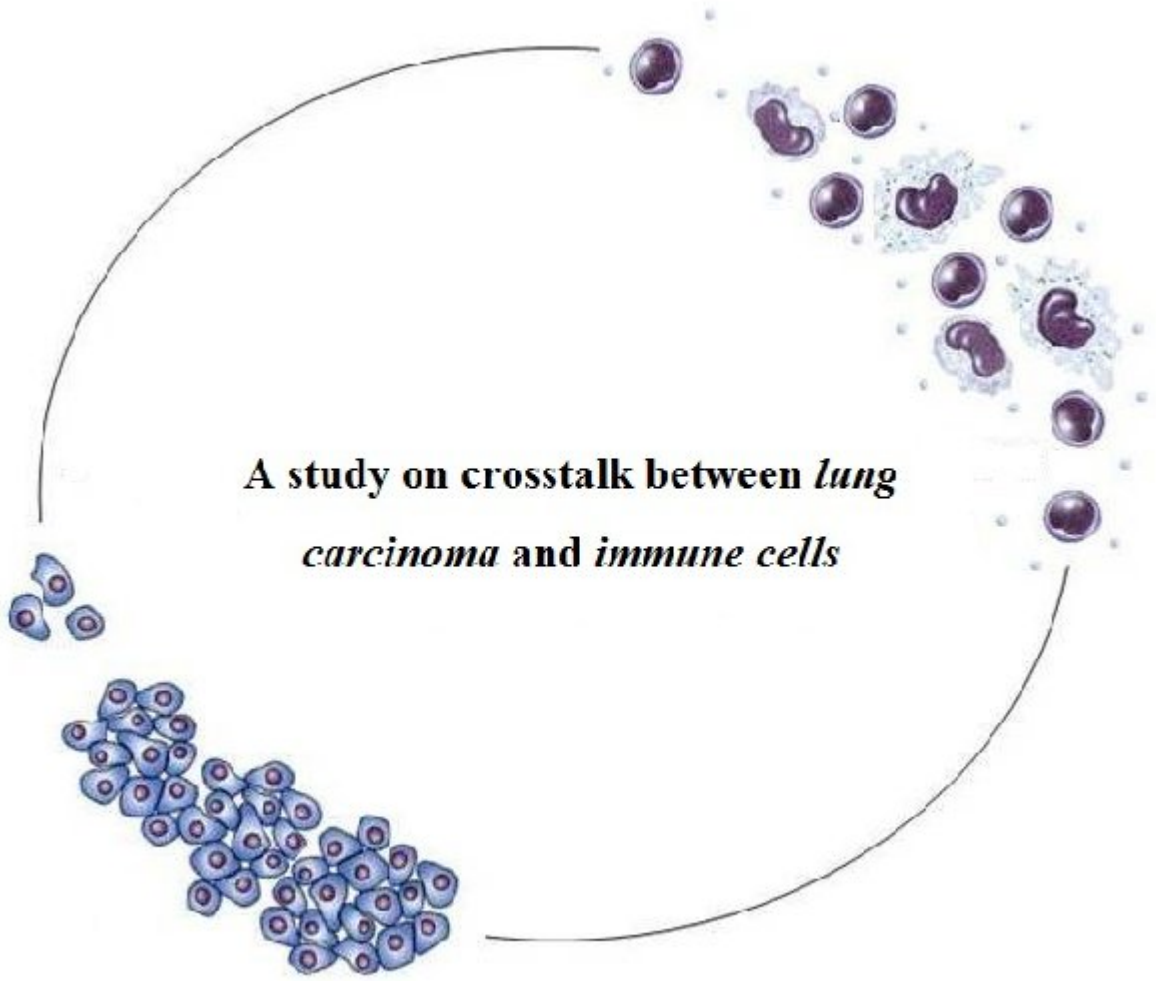

A study on crosstalk between *lung carcinoma* and *immune cells*

UNIVERSITY OF KASHMIR
DEPARTMENT OF BIOTECHNOLOGY

CERTIFICATE

This is to certify that the work contained in this thesis entitled **"A study on crosstalk between *lung carcinoma* and *immune cells*"** is the bonafide original research work of ***Mr. Qazi Danish Mushtaq*** and is worthy of consideration for the award of Doctor of Philosophy (Ph. D.) degree.

Dr. Raies Ahmad
Supervisor

Dr. Khalid Majid Fazili
Head of the Department

DECLARATION

The research work entitled: **"A study on crosstalk between *lung carcinoma* and *immune cells*"**, presented in this thesis, embodies the original research work done towards **Doctor of Philosophy** (Ph. D.) degree in the Department of Biotechnology at University of Kashmir, Srinagar. This work has not been submitted in part or in full for any other degree or diploma.

Qazi Danish Mushtaq

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Danish

CONTENTS

ABBREVIATIONS

APS	Ammonium per sulfate
ATCC	American type culture collection
BSA	Bovine serum albumin
CD	Cluster of differentiation
CRI	Cancer related Inflammation
CSF	Colony Stimulating factor
CXCL	C-X-C Motif Ligand
^{o}C	degree Celsius
DCs	Dendritic cells
DMA	N', N'-dimethyl acetamide
DMSO	Dimethyl sulphoxide
ECL	Enhanced Chemiluminescence
ECM	Extra Cellular Matrix
EDTA	Ethylenediamine tetra acetic acid
EGFR	Epithelial growth factor receptor
ELISA	Enzyme linked immunosorbent assay
EMT	Epithelial mesenchymal transition
ERK	Extracellular signal-regulated kinase
FCS	Fetal calf serum
Gr-1	Granzyme-1
h	hours
HIF	Hypoxia Inducing Factor
HPLC	High-performance liquid chromatography
HRP	Horse radish peroxidise
IFN	Interferon
Ig	Immunoglobulin
Iκ-B	Inhibitor of kappa B
IL	Interleukin
IP	Immunoprecipitation
Jak	Janus kinase or Just Another Kinase
LPS	Lipopolysaccharide
M	molar

MAPK	Mitogen-activated protein kinase
MDSC	Myeloid Derived Supressor cells
MHC	Major Histocompatibility Complex
min	minutes
MMP	Matrix metalloprotease
MP	Milk protein
MyD88	Myeloid Differentiating factor 88
MΦ	Macrophage
NC	Nitrocellulose
NF-κB	Nuclear factor-kappa B
NK cell	Natural Killer cell
NO	Nitric Oxide
OPD	Ortho-phenylene diamine
PAMP	Pattern Associated Molecular Pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PI	Propidium iodide
PMA	Phorbol 12-myristate 13-acetate
RPMI	Roswell Park Memorial Institute
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
STAT	Signal Transducer and Activator of transcription
TBABH	Tetra-butyl ammonium borohydride
TEMED	N, N, N', N'- Tetramethylethylenediamine
TGF	Transforming Growth Factor
Th cell	T helper cell
TIL	Tumor Infiltrating Lymphocyte
TLR	Toll like Receptor
TMB	3, 3', 5, 5' tetra methyl benzidine
TNF	Tumor Necrosis Factor
TRAIL	TNF related Apoptosis Inducing Ligand
T regs	T- regulator cells
VEGFR	Vascular Endothelial Growth Factor

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Tumor cells are seen to modulate the phenotype of all major immune cells to express tumor favouring phenotypes. Inflammation associated with tumors, a result of such interaction, is increasingly being believed to play a major role in tumor initiation, progression and even metastasis. This modulation is achieved very early when Monocytes, precursors of Macrophages and DCs, from the circulating pool are recruited towards tumors and selectively differentiated. Monocytes, in particular, are thought to generate a cytokine milieu in the microenvironment favourable to tumor. Such a crosstalk and the pathways involved therein are not well established, especially in human models. Using representative human carcinoma cells of different origin including Lung, Colon and Cervix, we show that factor(s) associated with these cells can activate secretion of tumor-associated cytokines, TNF-α, IL-6, IL-10, IL-12p40 but not IL-12p70 or IL-1β from human monocytes. Comparative murine co-cultures are also able to induce similar responses. Treatment of monocytes with TLR-2 blocking antibody inhibits these inflammatory responses upon encountering cell-associated as well as secretory ligand(s) from tumor cells. Pharmacological inhibition of intracellular MAP kinase pathway in carcinoma cells ablates the TLR-2 agonistic activity of carcinoma cells. However, inhibition of EGFR and Ras, two major oncogenic players, had no such effect. Early inflammatory response tends to enhance the proliferation and invasiveness of tumor cells and concurrently, increase the viability of monocytes. These tumor associated inflammatory responses may well be one of the mechanisms to manipulate effector T-cell response against tumors. These results suggest a previously unrecognized pathway that may regulate inflammatory responses triggered by cancer cells from monocytes. Our findings have important implications for understanding Cancer related Inflammation.

Carcinoma

Carcinoma is the medical term for the most common type of cancer occurring in humans. It is defined as a cancer that begins in a tissue that lines the inner or outer surfaces of the body, and that generally arises from cells originating in the endodermal or ectodermal germ layer during embryogenesis (Berman, 2004a). More specifically, a *carcinoma* is tumor tissue derived from putative epithelial cells, having the cytological appearance, histological architecture, or molecular characteristics of epithelial cells (Berman, 2004b) whose genome has become altered or damaged to such an extent that the cells become transformed, and begin to exhibit abnormal malignant properties.

Pathogenesis and Hallmarks of cancer

Cancer occurs when a single progenitor cell accumulates mutations and other changes in the DNA, histones, and other biochemical compounds that make up the cell's genome (Hanahan and Weinberg, 2001). Certain combinations of mutations in the given progenitor cell ultimately result in that cell (also called a cancer stem cell) displaying a number of abnormal, malignant cellular properties that, when taken together, are considered characteristic or hallmarks of cancer (Hanahan and Weinberg, 2001), including:

- the ability to continue to divide perpetually, producing an exponentially (or near-exponentially) increasing number of new malignant cancerous "daughter cells" (uncontrolled mitosis);
- the ability to penetrate normal body surfaces and barriers, and to bore into or through nearby body structures and tissues (local invasiveness);
- the ability to spread to other sites within the body (metastasize) by penetrating or entering into the lymphatic vessels (regional metastasis) and/or the blood vessels (distant metastasis) (Figure A).

If this process of continuous growth, local invasion, and regional and distant metastasis is not halted *via* a combination of stimulation of immunological defenses and medical treatment interventions, the end result is that the host suffers a continuously increasing burden of tumor cells throughout the body. Eventually, the tumor burden increasingly interferes with normal biochemical functions carried out by the host's organs, and death ultimately ensues. A progenitor carcinoma stem cell can be formed from any of a number

of oncogenic combinations of mutations in a totipotent cell, a multipotent cell,or a mature differentiated cell (Figure A).

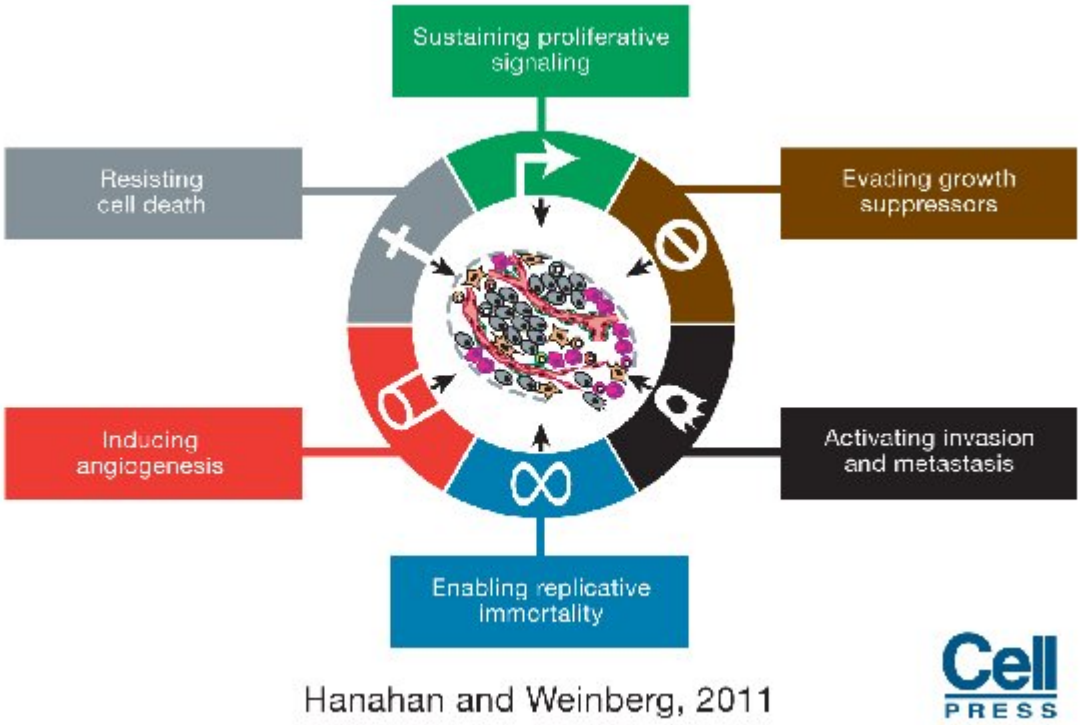

Figure A: Hallmarks of Cancer (developing cancer is centrally denoted).

Classification and types of carcinomas

Malignant neoplasms are exceptionally heterogeneous entities, reflecting the wide variety, intensity, and potency of various carcinogenic promoters. One commonly used classification scheme classifies these major cancer types on the basis of cell genesis, specifically, their (putative) cell (or cells) of origin (Travis *et al*., 2004)

1. Epithelial cells > *carcinoma*
2. Non-hematopoietic mesenchymal cells > *sarcoma*
3. Hematopoietic cells
   a) bone marrow-derived cells that normally mature in the bloodstream > *Leukemia*

b) bone marrow-derived cells that normally mature in the lymphatics > *Lymphoma*

4. Germ cells > *Germinoma*

Other criteria that play a role in a cancer classification, staging and diagnosis include the degree to which the malignant cells resemble their normal, untransformed counterparts, the appearance of the local tissue and stromal architecture., the anatomical location from which tumors arise and genetic, epigenetic, and molecular features.

Various histological types and variants of carcinoma are:

Adenocarcinoma: (*adeno* = gland) Refers to a carcinoma featuring microscopic glandular-related tissue cytology, tissue architecture, and/or gland-related molecular products, e.g., mucin.

Squamous cell carcinoma: Refers to a carcinoma with observable features and characteristics indicative of squamous differentiation (intercellular bridges, keratinization, squamous pearls).

Adenosquamous carcinoma: Refers to a mixed tumor containing both adenocarcinoma and squamous cell carcinoma, wherein each of these cell types comprise at least 10% of the tumor volume.

Anaplastic or Undifferentiated carcinoma: Refers to a heterogeneous group of high-grade carcinomas that feature cells lacking distinct histological or cytological evidence of any of the more specifically differentiated neoplasms.

Large cell carcinoma: Composed of large, monotonous rounded or overtly polygonal-shaped cells with abundant cytoplasm.

Small cell carcinoma: Cells are usually round and are less than approximately 3 times the diameter of a resting lymphocyte and little evident cytoplasm. Occasionally, small cell malignancies may themselves have significant components of slightly polygonal and/or spindle-shaped cells (Bermann, 2004b, Travis *et al*., 2004).

There are a large number of rare subtypes of *anaplastic, undifferentiated carcinoma*. Some of the more well known include the lesions containing pseudo-sarcomatous components: *spindle cell carcinoma* (containing elongated cells resembling connective tissue cancers), *giant cell carcinoma* (containing huge, bizarre, multinucleated cells), and *sarcomatoid carcinoma* (mixtures of spindle and giant cell carcinoma). *Pleomorphic carcinoma* contains spindle cell and/or giant cell components, plus at least a 10%

component of cells characteristic of more highly differentiated types (i.e. adenocarcinoma and/or squamous cell carcinoma). Very rarely, tumors may contain individual components resembling both *carcinoma* and true *sarcoma*, including *carcinosarcoma* and *pulmonary blastoma* (Travis *et al*, 2004)). Although tumors can arise in almost any tissue, the frequent organ sites of carcinoma are

- **Lung**: *Carcinoma* comprises >**98% of all lung cancers.**
- Breast: Nearly all breast cancers are *ductal carcinoma*.
- Prostate: The most common form of carcinoma of the prostate is *adenocarcinoma*.
- Colon and rectum: Nearly all malignancies of the colon and rectum are either *adenocarcinoma* or *squamous cell carcinoma*.
- Pancreas: C*arcinoma* is almost always of the adenocarcinoma type and is highly lethal.

Some carcinomas are named for their or the putative cell of origin, (e.g. *hepatocellular carcinoma, renal cell carcinoma*).

Cancer and the Immune System

The origins and progress of cancer immunology has been reviewed in depth, highlighting the development of ideas from Ehrlich and Medawar through to the cancer immune surveillance hypothesis of Burnet and into the era of cellular and molecular immunology (Dunn *et al*., 2002; Kaufmann, 2008). The immune system works essentially by discriminating self from non-self. Non-self is discriminated from self by fundamental differences in biochemistry, such as the arrangement of carbohydrate residues on glycoproteins or the absence of methylated cytosine residues in DNA. These differences are detected by the numerous pattern receptors, which are a hallmark of the innate immune system. These pattern receptors include the Toll-like receptors (O'Neill, 2008). The activation of innate immunity leads to the efficient priming of adaptive immune responses mediated by B and T cells. These cells carry antigen receptors and, through education and cooperation, can distinguish self from non-self antigen and trigger subsequent events. However, tumour cells are self in origin and their biochemistry and behaviour differs only subtly from their healthy counterparts and thus, requires the detection of altered self. There is now a substantial body of data to show that innate and acquired immune responses to tumours do exist and that a multitude of immune cell types and their associated molecules are involved in detecting and eliminating tumours.

Immunity to infection and tumour immunity share a common 'dark side', that of immune evasion. It is a sad fact that, by the time a patient presents with a clinically detectable tumour, the tumour has already successfully evaded cancer immune surveillance mechanisms and is living alongside the immune system. Indeed, the immune system places strong selective pressure on tumours (and pathogens). Ultimately, the rare tumour cells that have mutations in the pathways that allow immune detection, elimination and evasion, the phenomenon of Immunoediting, (Figure B) are the cells that survive, proliferate and kill the patient (Teng *et al*., 2008). The goal behind many immunotherapeutic strategies is to tip the balance from tumour immune evasion to a productive anti-tumour response.

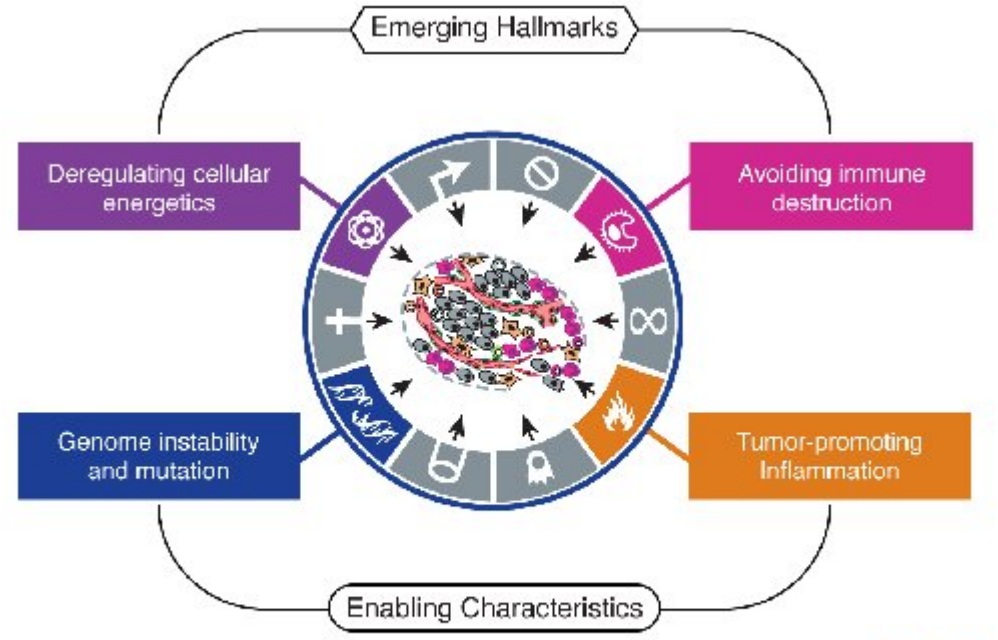

Figure B: Immune evasion and Inflammation (important components of *Immunoediting*) as Cancer Hallmarks. (from Hanahan and Weinberg, 2011).

Studies of the role of the cellular immune system in controlling cancer cells, promise to deliver not only fascinating insights into the immune system but also lay the foundation for future cellular immunotherapies. A better understanding of Tumor associated macrophages (TAM) and other myeloid-derived tumor-infiltrating cells as

pivotal players in the tumor microenvironment and as sources of Cancer-related inflammation (CRI) (Montovani *et al*., 2008) could certainly shed new light on the mechanistic understanding and development of efficient anticancer therapies. The present study was undertaken to understand the interaction of tumor cells of various origins, especially lung, with immune cells like monocytes/macrophages to establish new insights into such crosstalk.

1.1 Tumor Immunology

Tumor immunology is the study of interactions between the immune system and cancer cells (also called tumors or malignancies). It is also a growing field of research that aims to discover innovative cancer immunotherapies to treat and retard progression of this disease. An important role of the immune system is to identify and eliminate tumors. The *transformed cells* of tumors express antigens that are not found on normal cells. The immune response, including the recognition of cancer-specific antigens is of particular interest in this field as knowledge gained drives the development of new vaccines and antibody therapies. To the immune system, these antigens appear foreign, and their presence causes immune cells to attack the transformed tumor cells. The antigens expressed by tumors have several sources (Obeid *et al*., 2007) some are derived from oncogenic viruses like human papillomavirus, which causes cervical cancer (Zitvogel *et al*., 2004) while others are the organism's own proteins that occur at low levels in normal cells but reach high levels in tumor cells. The main response of the immune system to tumors is to destroy the abnormal cells using killer T cells, sometimes with the assistance of helper T cells. Tumor antigens are presented on MHC class I molecules of DCs' and Macrophages in a similar way to viral antigens. This allows killer T cells to recognize the tumor cell as abnormal. NK cells also kill tumorous cells in a similar way, especially if the tumor cells have fewer MHC class I molecules on their surface than normal; this is a common phenomenon with tumors. Sometimes antibodies are generated against tumor cells allowing for their destruction by the complement system. Clearly, some tumors evade the immune system and go on to become cancers. Tumor cells often have a reduced number of MHC class I molecules on their surface, thus avoiding detection by killer T cells (Green *et al*., 2009). Some tumor cells also release products that inhibit the immune response; for example by secreting the cytokine TGF-β, which suppresses activity of macrophages and lymphocytes (Bierie and Moses, 2006). In addition, immunological tolerance may develop against tumor antigens, so the immune system no longer attacks the tumor cells.

1.2 Immunosurveillance

There has been notable progress and accumulation of scientific evidence for the concept of cancer *immunosurveillance* and *immunoediting* based on (i) protection against development of spontaneous and chemically induced tumors in animal systems and (ii)

identification of targets for immune recognition of human cancer (Dunn *et al*, 2004). Cancer immunosurveillance is a theory formulated in 1957 by Burnet and Thomas, who proposed that lymphocytes act as sentinels in recognizing and eliminating continuously arising, nascent transformed cells (Dunn *et al.*, 2004; Smyth *et al.*, 2006). Cancer immunosurveillance appears to be an important host protection process that inhibits carcinogenesis and maintains regular cellular homeostasis (Kim *et al.*, 2007) It has also been suggested that immunosurveillance primarily functions as a component of a more general process of cancer immunoediting (Dunn *et al.*, 2002).

1.3 Immunoediting

Immunoediting is a process by which a person is protected from cancer growth and the development of tumour immunogenicity by their immune system. It has three main phases: elimination, equilibrium and escape (Kim *et al.*, 2007; Dunn *et al.*, 2004). The elimination phase consists of the following four phases:

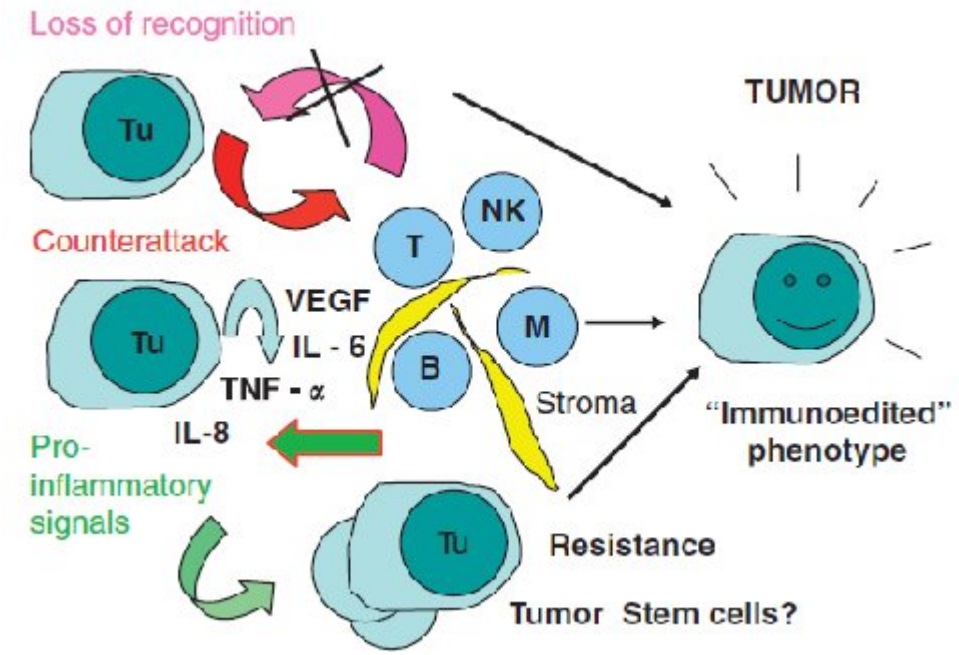

Figure 1.1: Mechanisms thought to be responsible for 'immunoediting' of tumor cells in the tumor microenvironment. (Whiteside, 2008)

***1.3.1 Elimination*: Phase 1**

The first phase of elimination involves the initiation of antitumor immune response. Cells of the innate immune system recognize the presence of a growing tumor which has undergone stromal remodeling, causing local tissue damage. This is followed by the induction of inflammatory signals which is essential for recruiting cells of the innate immune system (e.g. natural killer cells, natural killer T cells, macrophages and dendritic cells) to the tumor site (Figure 1.1). During this phase, the infiltrating lymphocytes such as the natural killer cells and natural killer T cells are stimulated to produce IFN-gamma (Zitvogel *et al.*, 2006).

***1.3.2 Elimination*: Phase 2**

In the second phase of elimination, newly synthesized IFN-gamma induces tumor death (to a limited amount) as well as promoting the production of chemokines CXCL10, CXCL9 and CXCL11. These chemokines play an important role in promoting tumor death by blocking the formation of new blood vessels. Tumor cell debris produced as a result of tumor death is then ingested by dendritic cells, followed by the migration of these dendritic cells to the draining lymph nodes. The recruitment of more immune cells also occurs and is mediated by the chemokines produced during the inflammatory process (Obeid *et al.*, 2007).

***1.3.3 Elimination*: Phase 3**

In the third phase, natural killer cells and macrophages transactivate one another via the reciprocal production of IFN-gamma and IL-12. This again promotes more tumor killing by these cells via apoptosis and the production of reactive oxygen and nitrogen intermediates. In the draining lymph nodes, tumor-specific dendritic cells trigger the differentiation of Th1 cells which in turn facilitates the development of CD8+ T cells.

***1.3.4 Elimination*: Phase 4**

In the final phase of elimination, tumor-specific CD4+ and CD8+ T cells home to the tumor site and the cytolytic T lymphocytes then destroy the antigen-bearing tumor cells which remain at the site.

1.3.5 Equilibrium and Escape

Tumor cell variants which have survived the elimination phase enter the equilibrium phase. In this phase, lymphocytes and IFN-gamma exert a selection pressure on tumor cells which are genetically unstable and rapidly mutating. Tumor cell variants which have acquired resistance to elimination then enter the escape phase. In this phase, tumor cells

continue to grow and expand in an uncontrolled manner and may eventually lead to malignancies (Figure 1.1). In the study of cancer immunoediting, knockout mice have been used for experimentation since human testing is not possible (Dunn *et al.*, 2004) Tumor infiltration by lymphocytes is seen as a reflection of a tumor-related immune response (Odunsi and Old, 2007).

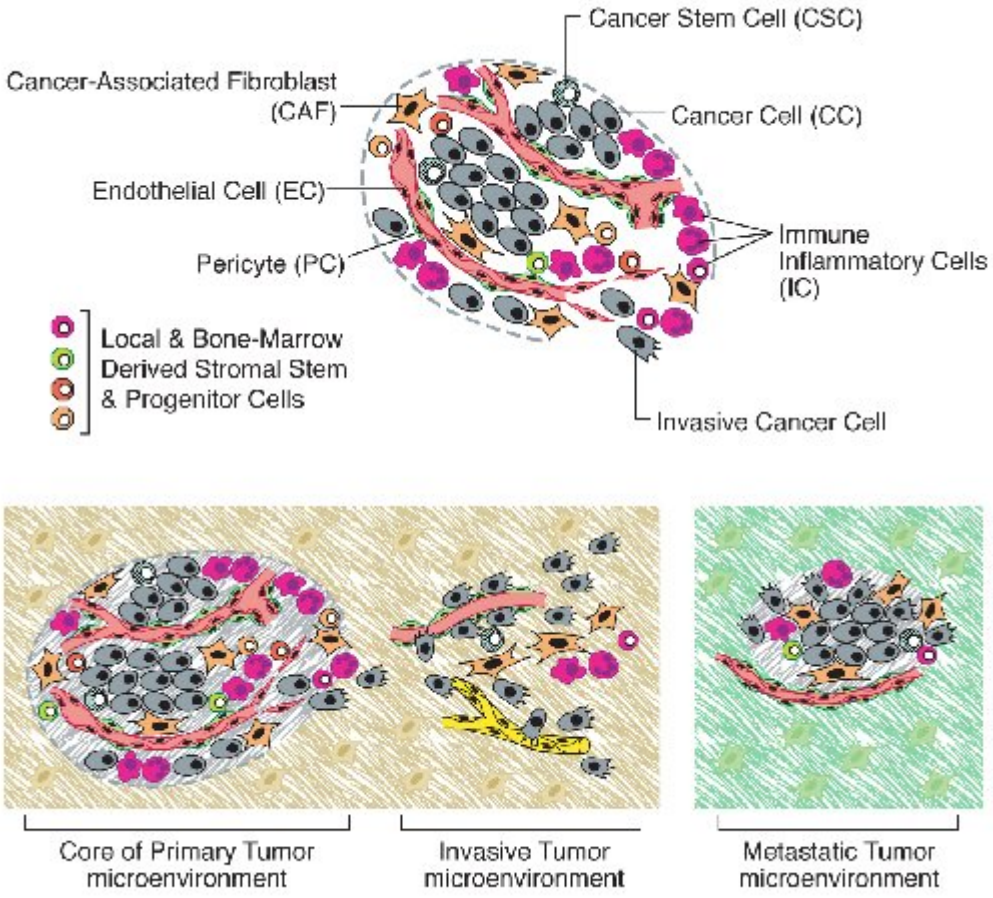

Figure1.2: The Cells of the Tumor Microenvironment (Hanahan and Weinberg. 2011).

1.4 Cells in the tumor microenvironment

A tissue microenvironment of developing tumor is comprised of proliferating tumor cells, the tumor stroma, blood vessels, infiltrating inflammatory cells and a variety of associated tissue cells (Figure 1.2). It is a unique environment that emerges in the

course of tumor progression as a result of its interactions with the host. It is created by and at all times shaped and dominated by the tumor, which orchestrates molecular and cellular events taking place in surrounding tissues. Immune cells present in the tumor include those mediating adaptive immunity, T-lymphocytes, dendritic cells (DC) and occasional B cells, as well as effectors of innate immunity, macrophages, polymorphonuclear leukocytes and rare natural killer (NK) cells (Whiteside, 2007).

1.4.1 Natural Killer (NK) Cells

NK cells, which mediate innate immunity and are rich in perforin- or granzyme-containing granules, are conspicuously absent from most tumor infiltrates or even pre-cancerous lesions (Whiteside *et al*., 1998). Although NK cells represent 'the first line' of defense against pathogens (Lanier, 2003) and mediate potent antitumor cytotoxicity in vitro, in tumor milieu, they are infrequent, despite the fact that tumor cells frequently downregulate expression of HLA antigens and are enriched in MICA and MICB molecules (Chang *et al*., 2005). These features make the tumor susceptible to NK cell-mediated cytotoxicity (Lee *et al*., 2004), and their paucity in tumor infiltrates may be an example of the evasion mechanism preventing NK-cell recruitment to the tumor site.

1.4.2 Tumor-infiltrating lymphocytes (TILs)

TILs, containing various proportions of CD3þCD4þ and CD3þCD8þ T cells, are usually a major component of the tumor microenvironment (Whiteside, 2007). Many of these T cells are specific for tumor-associated antigens, as indicated by clonal analyses (Miescher *et al*., 1987) and tetramer staining of CD8þ T cells isolated from human tumors (Albers *et al*., 2005). In some tumors, for example, medullary breast carcinomas, infiltrating lymphocytes form lymph node-like structures suggesting that the immune response is operating in situ (Coronella *et al*., 2002). Also, TILs are a source of tumor-specific lymphocytes used for adoptive transfers after expansion in IL-2-containing cultures (Zhou *et al*., 2004). TIL clones with the specificity to a broad variety of the tumor-associated antigens can be outgrown from human tumors, confirming that immune responses directed not only at 'unique' antigens expressed by the tumor, but also at a range of differentiation or tissue-specific antigens, are generated by the host (Romero *et al*., 2006). Although accumulations of these effector T cells in the tumor might be considered as evidence of immune surveillance by the host, they are largely ineffective in

arresting tumor growth. Among CD4þ T cells present in the tumor, a subset of CD4þCD25high Foxp3þ cells is expanded (5–15% of CD3þCD4þ T cells in TIL) relative to their significantly lower frequency in the peripheral circulation of patients with cancer (Woo *et al*., 2001; Strauss *et al*., 2007). These cells are regulatory T cells (Treg) capable of suppressing proliferation of other T cells in the microenvironment through contact-dependent mechanisms or IL-10 and TGF-b secretion. They come in different flavors (for example, nTreg, Tr1) and are a characteristic feature of the microenvironment in human tumors (Bergmann *et al*., 2007; Strauss *et al*., 2007).

1.4.3 Macrophages (MΦ)

Macrophages present in tumors are known as tumor associated macrophages or TAMs. Paradoxically, Macrophages can promote tumor growth (Pollard, 2004) when tumor cells send out cytokines that attract macrophages, which then generate cytokines and growth factors that nurture tumor development. In addition, a combination of hypoxia in the tumor and a cytokine produced by macrophages induces tumor cells to decrease production of a protein that blocks metastasis and thereby assists spread of cancer cells. They are re-programmed to inhibit lymphocyte functions through release of inhibitory cytokines such as IL-10, prostaglandins or reactive oxygen species (ROS) (Mantovani *et al*., 2005; Martinez *et al*., 2009). We discuss these cells in detail later.

1.4.4 Dendritic Cells

DCs are terminally differentiated myeloid cells that specialize in antigen processing and presentation. DCs differentiate in the bone marrow from various pro-genitors (Steinmann, 1991, Vermi *et al*., 2011). Monocytes are the major precursors of DCs in humans (Vermi *et al*., 2011; Lin *et al*., 2010). Two major subsets of DCs are currently recognized: conventional DCs (cDCs) and plasmacytoid DCs (pDCs). Although these cells share some common progenitors, their differentiation is controlled by distinct genetic programmes and they have different morphologies, markers and functions (Vermi *et al*., 2011; Shurin *et al*., 2006). The centrepiece of DC biology is the concept of functional activation and maturation in response to ‘dangerous’ stimuli. Differentiated DCs reside in tissues as ‘immature’ cells that actively take up tissue antigens but are poor antigen presenters and do not promote effector T cell differentiation. Only functionally activated DCs can effectively stimulate immune responses. DCs are activated in response

to stimuli associated with bacteria, viruses or damaged tissues; such stimuli are commonly referred to as pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs).

The fact that cancer can have profound effects on the function of DCs has been known for quite some time now. It is established that DCs in tumour-bearing hosts do not adequately stimulate an immune response, and this potentially contributes to tumour evasion of immune recognition. Evidence from numerous studies strongly indicates that abnormal myelopoiesis is the dominant mechanism responsible for DC defects in cancer (Shurin, 2012; Lotza, 1997). This abnormal differentiation produces at least three main results: decreased production of mature functionally competent DCs; increased accumulation of immature DCs at the tumour site; and increased production of immature myeloid cells (Lin *et al*., 2010; Shurin, 2012). In recent years, multiple clinical studies have confirmed the findings of earlier studies and have indicated that there is a decreased presence and defective functionality of mature DCs in patients with breast, non-small cell lung, pancreatic, cervical, hepatocellular or prostate cancer, or glioma (Poppena *et al*., 1983; Nestor and Cochran, 1987; Lijuna *et al*., 2012).

Some DCs in tumour-bearing hosts actively suppress T cell function, and both phenotypically immature and phenotypically mature DCs may be conditioned by the environment to support immune tolerance or immunosuppression (Lin *et al*., 2010; Shurin, 2012). MHC-II+CD11b+CD11c+ tumour-infiltrating mouse DCs have been shown to suppress CD8+ T cells and antitumour immune responses through arginase 1 (ARG1) production (Shrin, 2012; reichert *et al*., 2001) , an immunosuppressive mechanism previously attributed only to mouse tumour-associated macrophages (TAMs) and MDSCs . Human lung tumour cells can convert mature DCs into TGFβ-producing cells, and mouse lung cancer can drive DCs to express high levels of IL-10, nitric oxide, VEGF and ARG1 (Ladanyi *et al*., 2007; Sehrama *et al*., 2001; Reichert *et al*, 2001).

1.4.4 Myeloid suppressor cells (MSC)

MSC accumulating in human tumors are CD34þCD33þCD13þCD15(+) bone marrow-derived immature dendritic cells, an equivalent to CD11bþ/ Gr1þ cells in mice (Serafini *et al*., 2006). They promote tumor growth and suppress immune cell functions through copious production of an enzyme involved in L-arginine metabolism, arginase-1, which synergizes with iNOS to increase superoxide and NO production, blunting

lymphocyte responses (Ochoa *et al*., 2007) and by induction of iNOS in surrounding cells (Tsai *et al*., 2007). Relatively little is known about human MSC. A report describes expansion of CD14þHLA-DR+low myeloid-derived cells exerting immune suppression through TGF-b production in the peripheral circulation of patients with metastatic melanoma treated with GM-CSF-based vaccines.

Polymorphonuclear leukocytes are infrequently seen in infiltrates of human tumors, with the exception of nests of eosinophils that may be present in association with tumor cells in various squamous cell tumors, for example. In contrast, granulocytes tend to be a major cellular component of many murine tumor models (Loukinova *et al*., 2000). This disparity may be because of a different nature of infiltrates, which in humans are chronic rather than acute. Acute cellular responses may be long gone by the time human tumors are diagnosed, biopsied and examined.

1.5 Cancer and Monocytes/Macrophages

The tumor mass is undoubtedly a multifaceted show, where different cell types, including neoplastic cells, fibroblasts, endothelial, and immune-competent cells, interact with one another continuously. Macrophages represent up to 50% of the tumor mass, and they certainly operate as fundamental actors. Macrophages constitute an extremely heterogeneous population; they originate from blood monocytes, which differentiate into distinct macrophage types, schematically identified as M1 (or classically activated) and M2 (or alternatively activated) (Gordon, 2003; Montovani, 2002). It is now generally accepted that TAM have an M2 phenotype and show mostly pro-tumoral functions, promoting tumor cell survival, proliferation, and dissemination (Gordon and Taylor, 2005; Montovani, 2002). High levels of TAM are often, although not always, correlated with a bad prognosis, and recent studies have also highlighted a link between their abundance and the process of metastasis (). Macrophage infiltration was studied along tumor carcinogenesis in a mouse model of pancreatic cancer induced by the expression of oncogenic *KrasG12D*. Macrophage infiltration began very early during the preinvasive stage of disease and increased progressively (Lin, 2001). Moreover, gene-modified mice and cell-transfer experiments have confirmed the pro-tumor function of myeloid cells and of their effector molecules. On the other hand, low macrophage infiltration into the tumor mass correlates with the inhibition of tumor growth and metastasis development in different animal models (Wyckoff *et al*, 2007; Lin *et al*, 2006; Hiraoka, 2008). Lin *et al*.

demonstrated that when MMTV-PyMT mice, which spontaneously develop mammary tumors, were crossed with mice lacking monocytes/macrophages (op/op), the tumor growth and spread were reduced significantly. Accordingly, when cocultured with tumor cells, macrophages secrete substances that stimulate tumor cell proliferation. This countersense in which cells of the immunological system work against self is the result of several refined tumor capabilities to mould immature cells and to suppress anticancer cell activity (Pollard, 2009). Within the tumor mass, another myeloid cell population defined as MDSCs characterized by immune suppressive activity by being able to suppress T cell blastogenesis in tumor-bearing hosts has also been identified (Galina *et al*., 2006; Bronte *et al*., 2001; Sica and Bronte, 2007).

1.5.1 Dr. Jekyll and Mr. Hyde: The Macrophage Heterogeneity in Inflammation and Immunity

Blood monocytes are not fully differentiated cells and are profoundly susceptible to several environmental stimuli. When recruited into peripheral tissues from the circulation, monocytes could differentiate rapidly in distinct, mature macrophages and exert specific immunological functions. M-CSF is the main regulator of the survival, proliferation, and differentiation of mononuclear phagocytes, and many studies have also identified a role in the subsequent polarization phase for this factor (Gordon, 2003; Condeelis and Pollard, 2006). Macrophages can be divided schematically into two main classes in line with the Th1/Th2 dichotomy (Figure 1.3). M1 macrophages (classically activated cells) originate upon encounter with IFN-γ and microbial stimuli such as LPS and are characterized by IL-12 high and IL-23 production and consequent activation of polarized type-I T-cell response (Pixley and Stanley, 2004; Pollard, 2009), cytotoxic activity against phagocytozed microorganisms and neoplastic cells, expression of high levels of RO-I, and good capability as APCs. In general, M1 macrophages act as soldiers: they defend the host from viral and microbial infections, fight against tumors, produce high amounts of inflammatory cytokines, and activate the immune response (Martinez *et al*., 2009; Goerdt *et al*., 1999). On the other hand, distinct types of M2 cells differentiate when monocytes are stimulated with IL-4 and IL-13 (M2a), with immune complexes/TLR ligands (M2b), or with IL-10 and glucocorticoids (M2c) (Pollard, 2009; Mantovani *et al*., 2005). Hallmarks of M2 macrophages are IL-10^{high} IL-12^{low} IL-1ra ^{high} IL-1 decoyR^{high} production, CCL17 and CCL22 secretion, high expression of mannose,

scavenger and galactose-type receptors, poor antigen-presenting capability and wound-healing promotion. M2 cells are workers of the host: they promote scavenging of debris, angiogenesis, remodeling and repair of wounded/damaged tissues. Of note, M2 cells control the inflammatory response by down-regulating M1-mediated functions (Martnez *et al*., 2009; Mantovani *et al*., 2005). In addition, M2 macrophages are competent effector cells against parasitic infections. The loss of equilibrium of M1 and M2 cell number may lead to pathological events: an M1 excess could induce chronic inflammatory diseases, whereas an uncontrolled number of M2 could promote severe immune suppression (Martinez *et al*., 2009) (Figure 1.3).

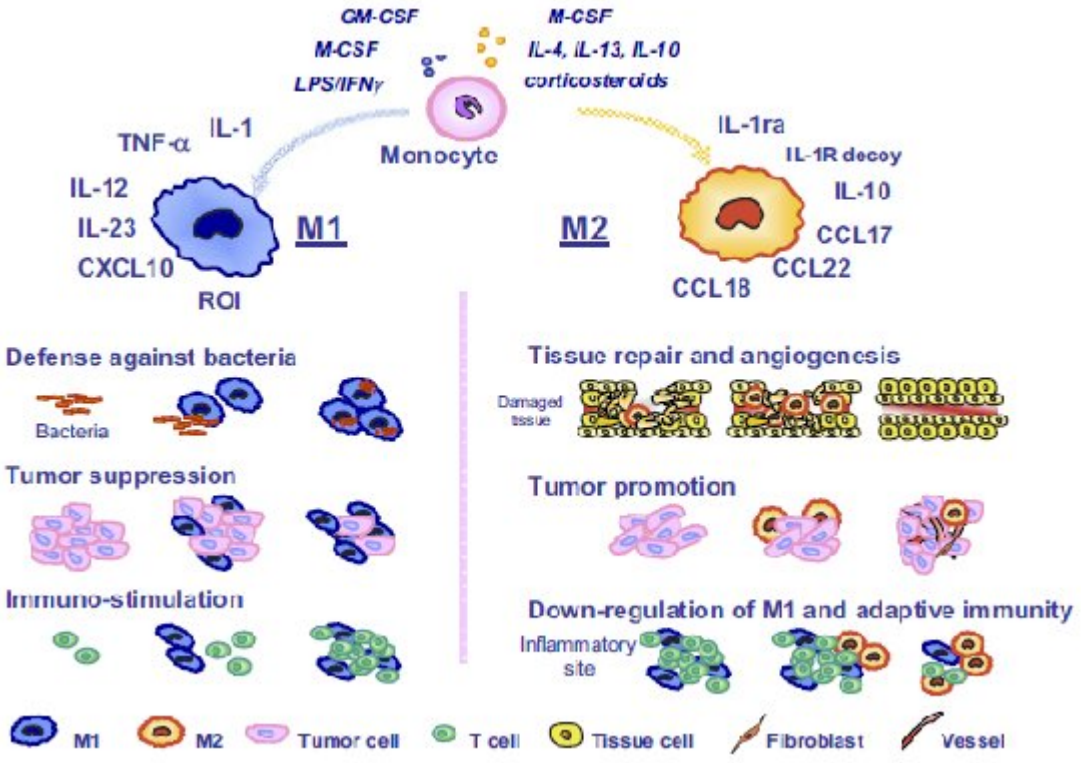

Figure 1.3: Polarization of macrophage function (Adapted from Allavena *et al*., 2009)

1.5.2 TAM-Tumor Associated Macrophage

TAMs originate from blood monocytes recruited at the tumor site (Pollard, 2004) by molecules produced by neoplastic and by stromal cells (Figure 1.4). The chemokine CCL2, earlier described in 1983 as a tumor-derived chemotactic factor, is the main player in this process (Allavena *et al*., 2008c; Pollard, 2004) and experimental and human studies correlate its levels with TAM abundance in many tumors, such as ovarian, breast and pancreatic cancer (Allavena *et al*., 2008c). TAM themselves produce CCL2, suggesting the action of an amplification loop and anti-CCL2 antibodies combined with other drugs have been considered as an anti-tumor strategy (Colombo and Mantovani, 2005). Other chemokines involved in monocyte recruitment are CCL5, CCL7, CXCL8, and CXCL12, as well as cytokines such as VEGF, PDGF and the growth factor M-CSF (Balkwill, 2004; Allavena *et al*., 2008c). Moreover, monocytes could be attracted by fibronectin, fibrinogen and other factors produced during the cleavage of ECM proteins induced by macrophage and/ or tumor cell-derived proteases (Denardo *et al*., 2008).

When monocytes (then macrophages) reach the tumor mass, they are surrounded by several signals able to shape the new cells as needed by the tumor (Figure 1.4). As far as they have been studied, TAM resemble M2-polarized macrophages (Mantovani *et al*., 2002; Pallard, 2004; Talmadge *et al*., 2007)]. This preferential polarization is a result of the absence of M1- orienting signals, such as IFN-γ or bacterial components in the tumor, as well as the expression of M2 polarization factors. In particular, the infiltration of Th2 lymphocytes (driven by Th2- recruiting chemokines such as eotaxins) has been reported in many tumors, and they are a fundamental source of IL-4 and IL-13 cytokines (Nevala *et al*., 2009; Cheadle *et al*., 2007). Moreover, neoplastic cells, fibroblasts, and Tregs produce TGF-β and IL-10. Incoming monocyte differentiation is also influenced by their localization within the tumor mass; for instance, in tumors, there is an established gradient of IL-10. This factor switches monocyte differentiation toward macrophages rather than DC (Cheadle *et al*., 2007; Li and Flavell, 2008)], and thus, as observed in breast cancer and in papillary carcinoma of the thyroid, TAM are present throughout the tissues, whereas DC are present only in the periphery (Scarpino *et al*., 2000).

The M2 polarization of TAM has also been demonstrated by studying their transcriptional profiling. Recent investigations noticed the up-regulation of many M2-associated genes such as CD163, Fc fragment of IgG, C-type lectin domains and heat shock proteins (Biswas *et al*., 2006; Sakai *et al*., 2008; Beck *et al*., 2009). In the tumor

milieu, TAM carry on their pro-neoplastic role by influencing fundamental aspects of tumor biology; they produce molecules that affect neoplastic cell growth directly (e.g., EGF), enhance neoangiogenesis, tune inflammatory responses and adaptive immunity and catalyze structural and substantial changes of the ECM compartment (Pollard, 2009; Mantovani *et al*., 2008; Allavena *et al*., 2008). Another hallmark of TAM is their tendency to accumulate into necrotic regions of tumors, characterized by low oxygen tension (Lewis and Murdoch, 2005). This preferential localization is regulated by tumor hypoxia, which induces the expression of HIF-1-dependent molecules (VEGF, CXCL12, and its receptor CXCR4) that modulate TAM migration in avascular regions (Talks *et al*., 2000; Schioppa *et al*., 2003)]. HIF-1 also regulates myeloid cell-mediated inflammation in hypoxic tissues (Cramer *et al*., 2003) and this link between hypoxia and innate immunity was confirmed recently, showing that HIF-1 is also regulated transcriptionally by NF-κB (Rius *et al*., 2008). Biochemical studies have identified the transcription factor NF-κB as a master regulator of cancer-related inflammation in TAM and in neoplastic cells. Constitutive NF-κB activation is indeed observed often in cancer cells and may be promoted by cytokines (e.g., IL-1 and TNF) expressed by TAM or other stromal cells, as well as by environmental cues (e.g., hypoxia and ROI) or by genetic alterations (Karin, 2006; Mantovani *et al*., 2008; Aggarwal, 2004). NF-κB induces several cellular modifications associated with tumorigenesis and more aggressive phenotypes, including self-sufficiency in growth signals, insensitivity to growth inhibition, resistance to apoptotic signals, angiogenesis, migration and tissue invasion (Pikarsky *et al*., 2004; Greten *et al*., 2004; Naugler and Karin, 2008). In a mouse model of colitis-associated cancer, the myeloid-specific inactivation of the Iκβ kinase inhibited inflammation and tumor progression, thus providing unequivocal genetic evidence for the role of inflammatory cells in carcinogenesis. On the other hand, in established, advanced tumors, where inflammation is typically smoldering (Balkwill *et al*., 2005), TAM usually have defective and delayed NFκ-B activation in response to different proinflammatory signals (e.g., expression of cytotoxic mediators such as NO, cytokines, TNF-α, and IL-12) (Biswas *et al*., 2006; Sica *et al*., 2000; Torroella-Kouri *et al*., 2005). These observations are in apparent contrast with a pro-tumor function of inflammatory reactions expressed by TAM. This discrepancy may reflect a dynamic change of the tumor microenvironment along tumor progression. In early stages of carcinogenesis, innate responses (inflammatory reactions) are indispensable for the activation of effective surveillance by

adaptive immunity (Dunn *et al*., 2004; Smyth *et al*., 2006) but on the other hand, are also likely to promote tumor development. In late stages of neoplasia, the defective NF-κB activation of TAM is insufficient to drive and sustain a potential anti-tumor immune response of the host. Evidence suggests that p50 homodimers (negative regulators of NF-κB) are abundant in TAM and are responsible for its defective activation (Saccani *et al*., 2006). As a matter of fact, TAM exert strong immune suppressive activity, not only by producing IL-10 but also by the secretion of chemokines (e.g., CCL17 and CCL22), which preferentially attract T cell subsets devoid of cytotoxic functions such as Treg and Th2 (Balkwill, 2004; Mantovani *et al*., 2004). In normal macrophages, these chemokines are inducible by IL-4, IL-10, and IL-13, thus amplifying an M2-mediated immune-suppressive loop. In addition, TAM secrete CCL18, which recruits naı¨ve T cells by interacting with an unidentified receptor (Schutyser *et al*., 2002). Attraction of naı¨ve T cells in a microenvironment characterized by M2 cells and immature DC is likely to induce T cell anergy.

1.5.3 TAM and Angiogenesis

Angiogenesis is sustained by different mediators produced by neoplastic and by stromal cells. TAM release growth factors such as VEGF, PDGF, TGF-β and members of the FGF family (Mantovani *et al*., 2002; Bingle *et al*., 2002), and the proangiogenic role is highlighted by the correlation between their high numbers and high vascular grades in many tumors such as glioma, squamous cell carcinoma of the esophagus, breast, bladder and prostate carcinoma (Bingle *et al*., 2002). TAM secrete the angiogenic factor thymidine phosphorylase, which in vitro promotes endothelial cell migration (Lin *et al*., 2006) and they also produce several angiogenesis modulating enzymes such as MMP-2, MMP-7, MMP-9, MMP-12, and cyclooxygenase-2 (Lin *et al*., 2006; Bingle *et al*., 2002).

1.5.4 TAM: Invasion and Metastasis

Metastasis unquestionably represents a crucial phase of neoplastic diseases and develops when tumor cells acquire specific capabilities to leave the primary tumor, invade the surrounded matrix, reach through blood or lymphatic vessels' distant sites, settle down and grow. As a result of its complexity, this process has yet to be analyzed further, but several lines of evidence have already identified a tight link between this process and TAM, which produce inflammatory cytokines likely active on the

dissemination stage. The intense cross-talk between macrophages and neoplastic cells guarantees the continuous process of matrix deposition and remodeling, which facilitates tumor growth and invasion of the surrounding tissues (Figure 1.4). The high tissue remodelling activity of TAM is summarized by Dvorak's definition: "Tumors are never healing wounds" (Dvorak, 1986; Codeelis and Pollard, 2006). TAM co-operate on tumor dissemination by promoting invasion characteristics of malignant cells and also by making easier their movement by a direct action on the tumor microenvironment (Hagemann *et al*., 2004). In particular, one of the main factor involved significantly is TNF-α: coculture of neoplastic cells with macrophages enhances invasiveness of malignant cells through TNF-dependent MMP induction in macrophages (Hagemann *et al*., 2004). TAM produce IL-1, and Giavazzi and colleagues (Giavazzi *et al*., 1990) demonstrated the IL-1-induced augmentation of metastasis development in a mouse melanoma model. In a genetic model of breast cancer growing in monocyte deficient mice, the tumors developed normally but in the absence of the macrophage-produced EGF, were unable to form pulmonary metastasis (Pollard, 2008).

1.5.6 TAM and Anti-Cancer Therapies

It is underlined how TAM favor neoplastic cells during tumor development and invasion and spread to distant sites. Thus, it is easy to gather that these cells may certainly be considered as an attractive target for novel anti-cancer therapies. If we block macrophages, will we actually disturb tumor progression in human patients? Within a tumor, a heterogeneous microenvironment differentially influences infiltrated macrophages, and this shows clearly the necessity of identifying common TAM targets for the synthesis of new therapeutic molecules (Zitvogel *et al*., 2008). Obviously, the best target would be a protein expressed or overexpressed only by TAM and neither by resident macrophages of distant, healthy tissues nor by M1 cells, which are important to face pathogens and could take part in anti-cancer actions. Several "anti-macrophage" approaches are under evaluation currently. Interesting observations come from studies performed with chemokines and chemokine receptors as anti-cancer targets (Zitvogel *et al*., 2008; Bingle *et al*., 2002).

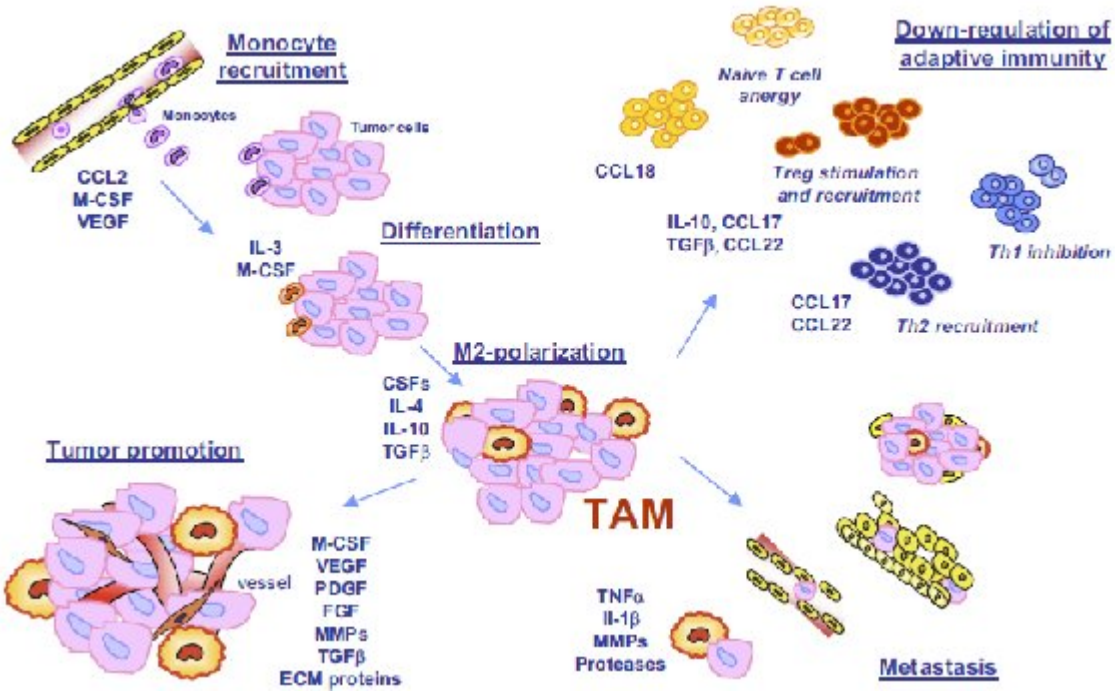

Figure 1.4: Overview of TAM, which originate from blood monocytes recruited at the tumor site by molecules produced by neoplastic and by stromal cells. (Adapted from Allavena et. al., 2009).

Macrophages have also been used to enhance the immune response or to potentiate chemotherapy specificity. Carta and colleagues (Carta *et al*., 2001) engineered a murine macrophage cell line that strongly augmented the production of IFN-γ. The delicate balance between M1 and M2 cells is a fundamental aspect in anti-cancer treatment also. Several studies have shown that the activation of TLRs (for instance, TLR9) stimulates M1-polarized macrophage responses by inducing the activation of a proinflammatory program (Krieg, 2006).

In general, the restoration of an M1 phenotype in TAM may provide a therapeutic benefit by promoting antitumor activities. SHIP1-deficient mice showed a skewed development toward M2 macrophages, and thus, pharmacological modulators of this phosphatase are under investigation currently (Ong *et al*., 2007; Guiducci *et al*., 2005).

Interestingly, a contribution of the immune system to the anti-tumor effects of conventionally used chemotherapy treatments has been suggested. Cells of the innate immunity can be activated by proteins secreted by dying cells— damage associated molecular patterns (Zitvogel *et al*., 2008; Green *et al*., 2009).

1.6 Cancer Related Inflammation (CRI)

The association between cancer and inflammation dates back to Rudolf Virchow (1863) when he noticed the presence of leukocytes in neoplastic tissues (Balkwill and Montovani, 2001). Studies have identified two main pathways linking inflammation and cancer: an intrinsic and an extrinsic pathway (Coussens and Werb, 2002). The first one includes genetic alterations that lead to inflammation and carcinogenesis, whereas the second one is characterized by microbial/ viral infections or autoimmune diseases that trigger chronic inflammation in tissues associated with cancer development. Both pathways activate pivotal transcription factors of inflammatory mediators (e.g., NF-κB, STAT3, and HIF-1) and inflammatory cells (Hagemann *et al*., 2008; Kin and Karin, 2007; Karin, 2006).

Inflammatory cells like DCs, Macrophages, Neutrophils etc. present in the tumor microenvironment either contribute to tumor progression or actively interfere with its development (Figure 1.5). It is clear now that the former takes precedence, largely because the tumor generally proceeds to establish mechanisms responsible for its 'immune evasion' or escape from the immune intervention (Talmadge *et al*., 2007). The tumor not only manages to escape from the host immune system, but it effectively contrives to benefit from infiltrating cells by modifying their functions to create the microenvironment favourable to tumor progression. To this end, immune cells infiltrating the tumor together with fibroblasts and extracellular matrix forming a scaffold supporting its expansion, contribute to establish an inflammatory milieu that nourishes the tumor and promotes its growth. Inflammation is a salutary response to insult or injury and an important part of innate immunity; however, chronic inflammation has been linked with the development of cancer. Individuals with *ulcerative colitis*, a chronic inflammatory disease of the colon, have a 10-fold higher likelihood of developing colorectal carcinoma. Similarly, inflammatory conditions of the liver, such as *chronic hepatitis* and *cirrhosis*, are well established risk factors for the development of hepatocellular carcinoma (Karin, 2006).

Chronic Inflammatory conditions have been observed in association with tumor incidence, tumor progression and detrimental prognosis in human cancer patients. It is still early to understand the molecular mechanisms of how and why tumors occur more frequently in an inflammatory microenvironment or in an inflammation-plagued host. Pro-inflammatory cytokines are not surprisingly at the crossroad of this deregulation. Several of these cytokines are highly expressed in human cancers and do alter the immune response in ways that are simultaneously beneficial to tumor growth (Kin and Karin, 2007). It is tempting to speculate that the observed derailing of antitumor immunity into an inflammatory response is at its core, a defensive strategy of the tumor, selected for independently of the tumor cell transformation. Alternatively, it might be the mere result of, and the default reaction to, the expression of transforming oncogenes within the tumor cell. Third, the presence of mutant cell clones in an inflamed and regenerating tissue could simply be an unfortunate coincidence. Here, the tumor cell would take advantage of the improved cytokine mediated growth conditions for the nascent tumor, whereas the same cytokines inhibit the immune-mediated tumor surveillance and tumor cell elimination (Dunn *et al*., 2002).

Recent research has highlighted an important role for inflammation in cancer from the perspective that innate immune cells, such as macrophages, drive malignant progression through the production of proinflammatory mediators such as tumor necrosis factor (TNF) and interleukin (IL)-6 (Greten *et al*., 2004; Maeda *et al*., 2003; Rakoff-Nahoum *et al*., 2004). In the context of gastric or colon cancer, the stimulus for activation of the innate immune cells may be provided by chronic infection with *Helicobacter pylori* or commensal bacteria that access the resident inflammatory cells through a breakdown in the barrier function of the epithelium during carcinogenesis. In cervical cancer and hepatocellular carcinoma, chronic infection with human papilloma virus (HPV) and hepatitis C virus (HCV), respectively, are clearly linked with carcinogenesis. The study by Naugler *et al*., using a mouse model of chemically induced liver cancer, suggests cell injury may also lead to the release of endogenous factors that activate innate immune cells. These authors showed that dead hepatocytes activate liver macrophages (Kupffer cells) through the molecule MyD88, which is an essential adaptor for Toll like receptor (TLR) signalling (Lawrence *et al*., 2007; Naugler and Karin, 2008). The TLRs are pathogen recognition molecules that are hard-wired to trigger activation of innate immunity upon recognition of pathogen-associated molecular patterns (PAMPs). TLRs

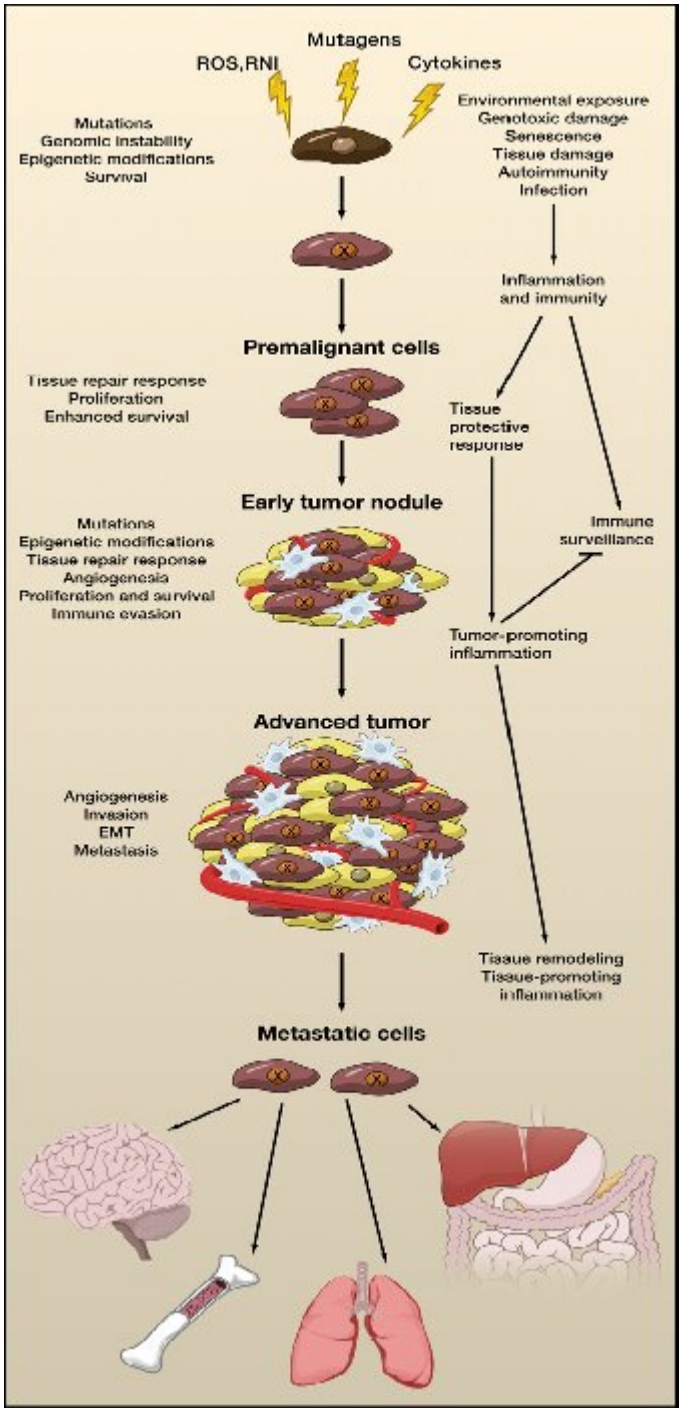

Figure 1.5: The Multifaceted Role of Inflammation in Cancer:
Inflammation acts at all stages of tumorigenesis. It may contribute to tumor initiation through mutations, genomic instability, and epigenetic modifications. Inflammation activates tissue repair responses, induces proliferation of premalignant cells, and enhances their survival. Inflammation also stimulates angiogenesis, causes localized immunosuppression, and promotes the formation of a hospitable microenvironment in which premalignant cells can survive, expand, and accumulate additional mutations and epigenetic changes. Eventually, inflammation also promotes metastatic spread. Mutated cells are marked with ‘‘X.’’ Yellow, stromal cells; brown, malignant cells; red, blood vessels; blue, immune and inflammatory cells. EMT, epithelial-mesenchymal transition; ROS, reactive oxygen species; RNI, reactive nitrogen intermediates.

have an important role in driving the inflammatory response but also in priming adaptive immunity through the activation and maturation of antigen presenting cells, including dendritic cells (DCs) and macrophages. Apetoh *et al*. (2007) have revealed an interesting role of inflammation and TLR signaling in cancer therapy.

The major antigen-presenting cells present in tumors are macrophages, which in certain cases may account for as much as 50% of the tumor mass; however, often it is not possible to detect an adaptive immune response to tumor antigens. There is increasing evidence that tumor associated macrophages (TAMs) express an immunosuppressive phenotype and display several protumoral functions, including promotion of angiogenesis and matrix remodelling (Balkwill *et al*., 2005; Pollard, 2004). Although usually rare, DCs have been detected in several tumor types, but DCs in tumors have been shown to express an immature phenotype and therefore to have low immunostimulatory properties (Mantovani *et al*., 2002). Both DCs and macrophages have the ability to pick up tumor antigens for crosspresentation on MHC class I molecules (Ardavin *et al*., 2004). However, the phenotype of TAMs and intratumoral DCs has been suggested to promote tolerance through production of immune- suppressive factors rather than prime a protective immune response (Mantovani *et al*., 2002).

1.7 Cytokines: The mediators of cancer and immune cell interplay

A solid body of evidence links increases in tumor incidence with inflammation. In addition, clinical and experimental findings also link tumor progression to the upregulation of pro-inflammatory molecules, particularly during the late stages of cancer progression and during tumor cachexia (Balkwill *et al*., 2005). Several of the cytokines linked to tumorpromoting inflammation such as tumor necrosis factor-a (TNF-a), transforming growth factor-b (TGF-b), IL-6 and IL-23 are functionally linked to the newly discovered Th17 CD4þ helper cell lineage (Balkwill, 2004).

1.7.1 Dual role for TNF-α in cancer

Tumor necrosis factor-α is a trimeric cytokine produced by activated macrophages and pro-inflammatory T cells. TNF-α can stimulate both pro-and antiapoptotic signals in tumor cells, endothelial cells, macrophages and most other cells within the tumor microenvironment (Szlosarek *et al*., 2006). TNF-α as well as IL-1 are essential effector cytokines for the initiation and maintenance of chronic inflammation in mouse models of

immune-mediated disorders such as rheumatoid arthritis (Williams *et al*., 2000). The relevance of this pathway for human disease is best exemplified in the success of anti-TNF-α therapies in inflammatory diseases (Feldmann and Maini, 2001). TNF-α also induces apoptosis in activated tumor-infiltrating T cells, and therefore may function to blunt the immune surveillance against tumors within the tumor itself. Although the pro-apoptotic effects of TNF-α spiked interest in its therapeutic utility, it requires higher concentrations than therapeutically achievable (Mocellin *et al*., 2005). Most animal models and clinical studies revealed the pro-neoplastic functions of TNF-α rather than its pro-apoptotic functions on tumor cells.

Tumor necrosis factor-a produced by tumor cells or inflammatory cells may promote tumor survival via the induction of antiapoptotic genes controlled by nuclear factor-kB activation. Indeed, TNF-α has been demonstrated to promote tumorigenesis as TNF-α-deficient mice or mice treated with anti-TNF-α antibodies are largely protected from the chemical induction of skin papillomas (Moore *et al*., 1999; Scott *et al*., 2003). TNF-α may also directly contribute to neoplastic transformation by stimulating production of genotoxic reactive oxygen species and nitric oxide (Szlosarek *et al*., 2006). In humans, higher concentrations of TNF-α are found in the serum of cancer patients compared to control subjects, and elevated TNF-α concentrations in the serum also correlate with decreased prognosis for the patients (Szlosarek and Balkwill, 2003). Finally, TNF-α is closely associated with tumor-induced cachexia, an inflammatory multiorgan failure in the late stage of cancer patients, and with the inflammatory paraneoplastic syndromes associated with tumors like pancreatic cancer. Genetic polymorphisms conferring higher TNF-α production are associated with increased risk of a variety of human cancers (Szlosarek *et al*., 2006). Excitingly, renal cell cancer patient treated in a phase II clinical study with anti-TNF-α antibodies experienced clinical benefits (Harrison *et al*., 2007).

1.7.2 Inflammation control by TGF-β

Another key regulator of inflammatory processes tightly associated with chronic inflammation and cancer is TGF-β. Although considered to be primarily antiinflammatory, TGF-β contributes to the inflammatory milieu of tumor mediators and cell types facilitating tissue remodeling as well as direct local suppression of antigen-specific CD8-T cell function. Transforming growth factor-β is a pleiotropic cytokine that

exerts effects on most cell types in a tumor thereby simultaneously impacting immunological and non-immunological processes. TGF-β activates a heterodimeric receptor pair of TGF-β receptors I and II (TbRI/II). Upon ligand binding, TbRI directly phosphorylates the transcription factors Smad2 and Smad3, which shuttle to the nucleus to induce transcription (Letterio, 2005). TGF-βb is released not only by a variety of cells in human and mouse tumors including macrophages, platelets and T cells (Kehrl *et al*., 1986; Roberts *et al*., 1986), but also by the tumor cells themselves. Early in the development of cancer and in premalignant lesions, TGF-β plays a tumor suppressive function due to its inhibition of tumor cell growth. Genetic deletion of the TGF-β receptor in genetic mouse models for human cancer leads to increased tumor incidence and progression (Bierie and Moses, 2006). Interestingly, ablation of TGF-β signaling in the tumor cells leads to an increased level of TGF-β in the tumor and increased number of TGF-β-producing $CD11b^{+}$ $GR\text{-}1^{+}$ myeloid cells in the tumor stroma (Yang *et al*., 2008). However, most human tumors thrive in the presence of large amounts of TGF-β while retaining the TGF-β signaling pathways. The exception appears to be malignancies of the gastrointestinal tract where mutations in either the TGF-β receptor or the Smads render the tumor cells insensitive to abundant TGF-β (Derynck *et al*., 2001). Autocrine TGF-β regulation in tumor cells plays an important role during invasion, metastasis and epithelial–mesenchymal transition of tumor cells (Oft *et al*., 2002). In addition, many of the tumors promoting effects of TGF-β involve paracrine regulation of inflammation and tissue remodeling. TGF-β modifies the activities of fibroblasts, endothelial cells, macrophages and T cells to engender an inflammatory milieu similar to chronic inflammatory diseases but deficient in cytotoxic cells such as CD8T cells and natural killer cells. TGF-β is one of the first proteins released from platelets after a vascular lesion, induces angiogenesis (Roberts *et al*., 1986) and is a potent chemoattractant for granulocytes and monocytes (Wahl *et al*., 1987; Brandes *et al*., 1991); TGF-β also limits the phagocytic and opsonizing activity of those innate responders. More importantly, although TGF-β promotes the development of Langerhans cells and dendritic cells from hematopoietic progenitors (Borkowski *et al*., 1996; Strobl *et al*., 1996), it inhibits the maturation, antigen presentation and costimulation by both macrophages and dendritic cells (Li *et al*., 2006).

Such immature dendritic cells produce large amounts of TGF-β and might efficiently prime regulatory CD4 T cells (Treg). TGF-β is required for the development of

Tregs, and TGF-β expression by Tregs is essential for their proliferation and function. Regulatory T cells are found in human tumors and their presence correlates again with a poorer prognosis (Curiel *et al*., 2004). In a new twist of the development of helper T-cell lineages, it has become clear that pro-inflammatory IL-17-producing Th17T cells share a common path with regulatory T cells. Although the presence of TGF-β favors a regulatory fate of naive T cells, simultaneous presence of TGF-β and IL-6 fosters the differentiation of a proinflammatory T cell expressing IL-6 and IL-17 among other cytokines (Bettelli *et al*., 2006). Transforming growth factor-β not only restricts the proliferation of naive CD4þ T cells by suppressing IL-2 production in T cells but also antagonizes both Th1 and Th2 effector differentiation (Li *et al*., 2006). At the same time, however, TGF-β protects T cells from apoptosis during T-cell expansion and differentiation. In particular, TGF-β inhibits activation-induced cell death of T cells (Zhang *et al*., 1995). Polyclonal T-cell activation in mice using activating anti-CD3 antibodies leads to widespread apoptosis of both $CD4^+$ and $CD8^+$ T cells in TGF-β1-/- mice (Chen *et al*., 2001). Similar to helper T cells, $CD8^+$ cytotoxic T cells are inhibited in their proliferation and differentiation by TGF-β (Wrzesinski *et al*., 2007). TGF-β inhibits the expression of cytokines like interferon-γ (IFN-γ) and cytotoxic effector molecules such as perforin, and also the exocytosis of the cytotoxic granules (Li *et al*., 2006).

Moreover, when stimulated with both IL-6 and TGF-β, CD8T cells not only cease expression of IFN-γ and lose their cytotoxicity but are also induced to secrete IL-17 (Liu *et al*., 2007). IFN-γ induces major histocompatibility complex I in both dendritic cells and tumor cells; therefore, replacing IFN-γ with IL-17 in the tumor milieu might have severe consequences for immune recognition and surveillance.

1.7.3 The pro-inflammatory cytokine IL-6 promotes tumor growth

Interleukin-6 engages the heterodimeric receptor complex of glycoprotein 130 (gp130) and IL-6 receptor-a (IL-6Ra). While gp130 is expressed in the signal receiving cell, the IL-6Ra subunit can be either membrane bound or supplied as a soluble receptor (sIL-6Ra) by an accessory cell, via a process known as trans-signaling (Rose-John *et al*., 2006). IL-6 induces the phosphorylation of both STAT3 and STAT1. The involvement of both IL-6 and STAT3 in malignant cell survival and proliferation has been well documented in numerous experimental systems (Aggarwal *et al*., 2006; Rose-John *et al*.,

2006). Through the activation of genes involved in cell cycle progression and suppression of apoptosis, IL-6 can directly protect tumor cells from apoptosis. IL-6 has also been shown to act as an autocrine growth factor for tumors (Baffet *et al*., 1991). IL-6 is essential in the initiation and maintenance of chronic inflammation of the colon (Atreya *et al*., 2000). Trans-signaling of IL-6 is similarly essential for the development of inflammation-induced colon tumors (Becker *et al*., 2004). Finally, antibody-mediated inhibition of IL-6 delays the development of chemically induced colitis-associated colon cancer (Becker *et al*., 2004). Interleukin-6 levels are elevated in the serum and tissue of cancer patients with multiple myeloma, renal cell, ovarian, colon, breast or prostate cancers. The IL-6 serum levels correlate negatively with the prognosis in breast and prostate cancer patients (Smith *et al*., 2001; Rao *et al*., 2006). IL-6Ra is highly expressed on tumor cells, with some evidence for shedding of the sIL-6Ra to stimulate trans-signaling in cells not expressing IL-6Ra (Becker *et al*., 2004; Rose-John *et al*., 2006). IL-6 may also be a cancer-predisposing genetic risk factor, with IL-6 promoter polymorphisms leading to higher IL-6 expression leading to a worse prognosis for colon cancer patients (Landi *et al*., 2003).

In combination with TNF-a, IL-6 stimulates the expansion and cytotoxicity of naive CD8T cells in vitro (Sepulveda *et al*., 1999); however, IL-6Ra has been shown to be downregulated upon activation in naïve and memory T cells (Betz and Muller, 1998), suggesting that its potential stimulatory effect on tumor-infiltrating effector lymphocytes may be lost. Recently, however, it has become clear that IL-6 together with TGF-b is crucial for the induction of IL-17-producing Th17 helper cell lineage (Mangan *et al*., 2006; Wilson *et al*., 2007). It remains to be tested how many of the effects of IL-6 in the regulation of tissue inflammation and cancer are dependent on the induction and subsequent control of this T-cell lineage. Importantly, it has been shown that the pro-inflammatory T helper cells continue to express both IL-17 and IL-6 (Becker *et al*., 2004; Langrish *et al*., 2005). In inflammatory disease models, deficiency of IL-17 ameliorates the disease, deficiency of both IL-6 or IL-23, a cytokine controlling the activity of Th17 cells, protected animals from disease (Alonzi *et al*., 1998; Cua *et al*., 2003; Nakae *et al*., 2003). While IL-6-deficient animals show a partial resistance to chemical-induced skin tumors (Ancrile *et al*., 2007); the absence of IL-23 renders animals completely protected from tumors (Langowski *et al*., 2006).

1.7.4 Interleukin 10

Another cytokine that activates STAT3 is IL-10 (Moore *et al*., 2001)). However, the effects of IL-10 are dramatically opposed to those of IL-6, as IL-10 is immunosuppressive and anti-inflammatory (Allavena *et al*., 1998; Moore *et al*., 2001). IL-10 inhibits NF-kB activation through ill-defined mechanisms (Mocellin *et al*., 2001; Moore *et al*., 2001) and consequently inhibits the production of proinflammatory cytokines, including TNF-α, IL-6, and IL-12 (Vicari and Tinchieri, 2004). Given this, it is no wonder that IL-10 inhibits tumor development and progression. The most striking effects of IL-10 are seen in Il10–/– mice, which are more prone to colonic inflammation and CAC when chronically infected with certain enteric bacteria, such as Helicobacter hepaticus (Akdis and Blaser, 2001). The mechanisms responsible for IL-10 inhibition of colitis are not completely clear but might be linked to its ability to counteract IL-12–driven inflammation or its ability to inhibit NF-kB activation (Sato *et al*., 2011). Indeed, enhanced IL-12p40 production by immune cells is a key feature of colonic inflammation Suppression of TNF-α and IL-12 release by DCs and macrophages might also contribute to the antitumor activity of Tregs and IL-10 (Allavena *et al*., 1998; Moore *et al*., 2001). However, it is not clear how STAT3 activation by IL-10 results in an antitumor effect, whereas STAT3 activation by IL-6 is considered to be pro-tumorigenic. Studies also suggest that IL-10 possesses immunostimulatory activity that enhances antitumor immunity (Mocellin *et al*., 2004). Although IL-10 usually exerts antitumor activity, its biological effects are not all that simple, and consistent with its ability to activate STAT3, it might also promote tumor development. Direct effects of IL-10 on tumor cells that might favor tumor growth have been reported. For example, an IL-10 autocrine and/or paracrine loop might have an important role in tumor cell proliferation and survival. IL-10 has also been shown to modulate apoptosis and suppress angiogenesis during tumor regression (Sato *et al*., 2011). Expression of IL-10 in mammary and ovarian carcinoma xenografts inhibits tumor growth and spread (Sato *et al*., 2011). IL-10 has complex effects on tumor development. In many experimental systems, IL-10 is found to exert antitumor activity, but in other cases it can be pro-tumorigenic (Sato *et al*., 2011). These dramatically opposing effects of IL-10 might depend on interactions with either cytokines or factors found in the tumor microenvironment, as it is unlikely that IL-10 functions in isolation. A better understanding of IL-10 signaling is needed before its effects on tumor growth and antitumor immunity can be fully explained.

1.7.5 Inflammation control by IL-23 and IL-12 in cancer

Interleukin-23 and IL-12 are closely related heterodimeric pro-inflammatory cytokines with similar structures, similar cellular sources and cellular targets but opposing functions. They are composed of a shared p40 subunit, structurally related to cytokine receptors, and a unique subunit, IL-23p19 or IL-12p35, structurally four-helix bundle cytokines (Kastelein *et al*., 2007). IL-12 uses the heterodimeric receptors IL-12Rb1 and IL-12Rb2, whereas IL-23 activates IL-12Rb-1/IL-23R dimers (Parham *et al*., 2002). Like IL-12, IL-23 induces TYK2-and JAK2-mediated phosphorylation of the transcription factors STAT1, STAT3 and STAT5, the phosphorylation of STAT4 being to a lesser extent (Parham *et al*., 2002; Trinchieri, 2003). The receptors for both IL-12 and IL-23 are primarily expressed on T, natural killer and natural killer T cells, with low levels present on monocytes, macrophages and dendritic cells. Both cytokines are produced primarily by activated antigen-presenting cells in response to bacterial products (Trinchieri *et al*., 2003). Consequently, IL-12p40-deficient mice, lacking IL-12 and IL-23, are highly susceptible to numerous bacterial, fungal and parasite infections including Salmonella, Citrobacter, Cryptococcus and Leishmania species (Bowman *et al*., 2006). For the response against most of these pathogens, IL-12- mediated responses are essential, whereas the IL-23 contribution is often only detected in the simultaneous absence of IL-12 (Kastelein *et al*., 2007). Instant lethal doses of Klebsiella or Citrobacter, however, require IL-23-mediated host responses in mice (Happel *et al*., 2003; Mangan *et al*., 2006). Surprisingly, these susceptibilities have not been described for IL-12p40- or IL12Rb1-deficient humans who suffer exclusively from mycobacterial and salmonella infection but show normal resistance to most other pathogens, including viruses (Novelli and Casanova, 2004).

IL-12 treatment in preclinical tumor models promotes immune surveillance against transplanted syngeneic tumors by inducing IFN-g-producing Th1 cells and the proliferation and cytotoxic activity of $CD8^+$ T cells and natural killer cells. IL-12-induced IFN-γ is not only rate limiting for T-cell activity but also induces the expression of major histocompatibility complex I and thereby allows increased recognition of tumor antigens (Wong *et al*., 1984). Tumor immune surveillance in mouse models is largely dependent on IFN-γ-expressing T cells (Kaplan *et al*., 1998). Similar experiments using IL-23 expressed in the transplanted tumor cell or systemically were equally efficient in rejecting syngeneic transplanted tumors (Lo *et al*., 2003).

Interleukin-12 is therefore generally considered to promote antitumor effects, and cancer patients have been treated with recombinant IL-12 in several clinical studies (Atkins *et al*., 1997). Dose-limiting toxicities were, however, observed before clinical benefits had been achieved. The toxicities appeared to be IFN-γ associated and were most likely the manifestations of a systemic immune response. Subsequent attempts combining IL-12 therapy with a peptide vaccine have so far not revealed enhanced clinical benefits in the IL-12 treatment arms (Cebon *et al*., 2003). The difference in IL-12 and IL-23 function against bacteria or tumors in mice and in man might be a reflection on the amount of infectious particles or the antigenic dose challenging the host defense. Most mouse models frequently use systemic exposure of the host to millions of colony-forming units of bacteria and viruses or injections with large numbers of tumor cells. Immune recognition of human tumors might, however, follow a quite different kinetic, with only limited antigen exposure at first. The majority of infections in human patients are similarly not characterized by initial exposure to large numbers of infectious particles. However, there are also striking differences in the regulation of immune surveillance to tumors in either IL-12- or IL-23-deficient animals. IL-12 deficiency increases not only the incidence of tumors but also allows for rapid tumor growth in mice. In contrast, deficiency in IL-23 or the IL-23 receptor not only dramatically reduces tumor incidence but also reduces tumor growth of established tumors (Langowski *et al*., 2006). In the local tumor microenvironment, IL-23 not only induces the hallmarks of chronic inflammation such as metalloproteases, angiogenesis and macrophage infiltration, but also reduces antitumor immunosurveillance by locally suppressing the presence of CD8-T cells.

In contrast, the absence of IL-12 leads to exacerbation of the myeloid-driven inflammation with a coincident lack of CD8 T cells (Langowski *et al*., 2006). Interestingly, it is IL-23p19 and IL12p40 that are found to be overexpressed in the majority of human cancers, not IL-12p35. In mouse models of autoimmune diseases, IL-23 induces chronic inflammation in part through the stimulation of innate myeloid effector cells and stromal activation, and many aspects of IL-23-dependent tissue inflammation can be recapitulated in the absence of T cells (Uhlig *et al*., 2006). However, IL-23 also controls the activity of Th17 T cells. Although Th17 develop from naive T cells under the influence of TGF-b and IL-6, they subsequently require IL-23 to suppress endogenous IL-10 and become proficient in their pro-inflammatory function (McGeachy *et al*., 2007). This pro-inflammatory function orchestrates inflammatory tissue destruction

by the adaptive immune system. The induction of IL-17 by IL-23 in tumors is an attractive prospect because IL-17 promotes angiogenesis in a variety of models and induces matrix metalloproteinases, two events that potentiate tumor growth (Numasaki *et al*., 2003).

In addition, IL-17 controls neutrophil chemotaxis, proliferation and maturation further fueling the innate immune activation (Kolls and Linden, 2004). IL-17 producing CD8 and CD4 T cells have recently been reported to be widely present in human and mouse tumor microenvironments (Kryczek *et al*., 2007). It has also been suggested that CD8 T cells expressing IL-17 largely lack cytotoxic capacity (Liu *et al*., 2007). It is important to note that IL-23 can induce, independent of IL-17, angiogenic erythema, inflammation and keratinocyte hyperproliferation, phenocopying aspects of human psoriatic lesions (Chan *et al*., 2006). Psoriatic disease does not correlate with increased incidence of malignancy (Rohekar *et al*., 2008). The IL-23-mediated physiological changes in the skin, however, strikingly resemble the microenvironment observed in early malignant lesions.

1.7.6 IL-1

Interleukin-1 is a pleiotropic cytokine that affects mainly inflammation and also contributes to immune and hemopoietic responses (Apte and Voronov, 2002; Dinarello, 1996). The properties of IL-1 stem from its ability to induce the synthesis of cytokines, chemokines, proinflammatory molecules, and the expression of adhesion molecules. The IL-1 gene family consists of two major agonistic molecules, namely IL-1α and IL-1β, and one antagonistic cytokine, the IL-1R antagonist (IL-1Ra). IL-1α, IL-1β, and IL-1Ra are encoded by different genes. Both IL-1α and IL-1β differ from most other cytokines by lacking a signal sequence, thus not trafficking through the endoplasmic reticulum (ER)-Golgi pathway; the precise mechanisms of IL-1 secretion are thus largely unknown (Apte and Voronov, 2002). IL-1α and IL-1β bind to the same receptors, and there are no significant differences in the spectrum of activities of recombinant IL-1α or IL-1β when studied in vitro or in vivo in diverse experimental systems. However, endogenously produced IL-1α and IL-1β differ dramatically in the subcellular compartments in which they are active. IL-1α is active in its secreted form (17.5 kDa), whereas the IL-1α

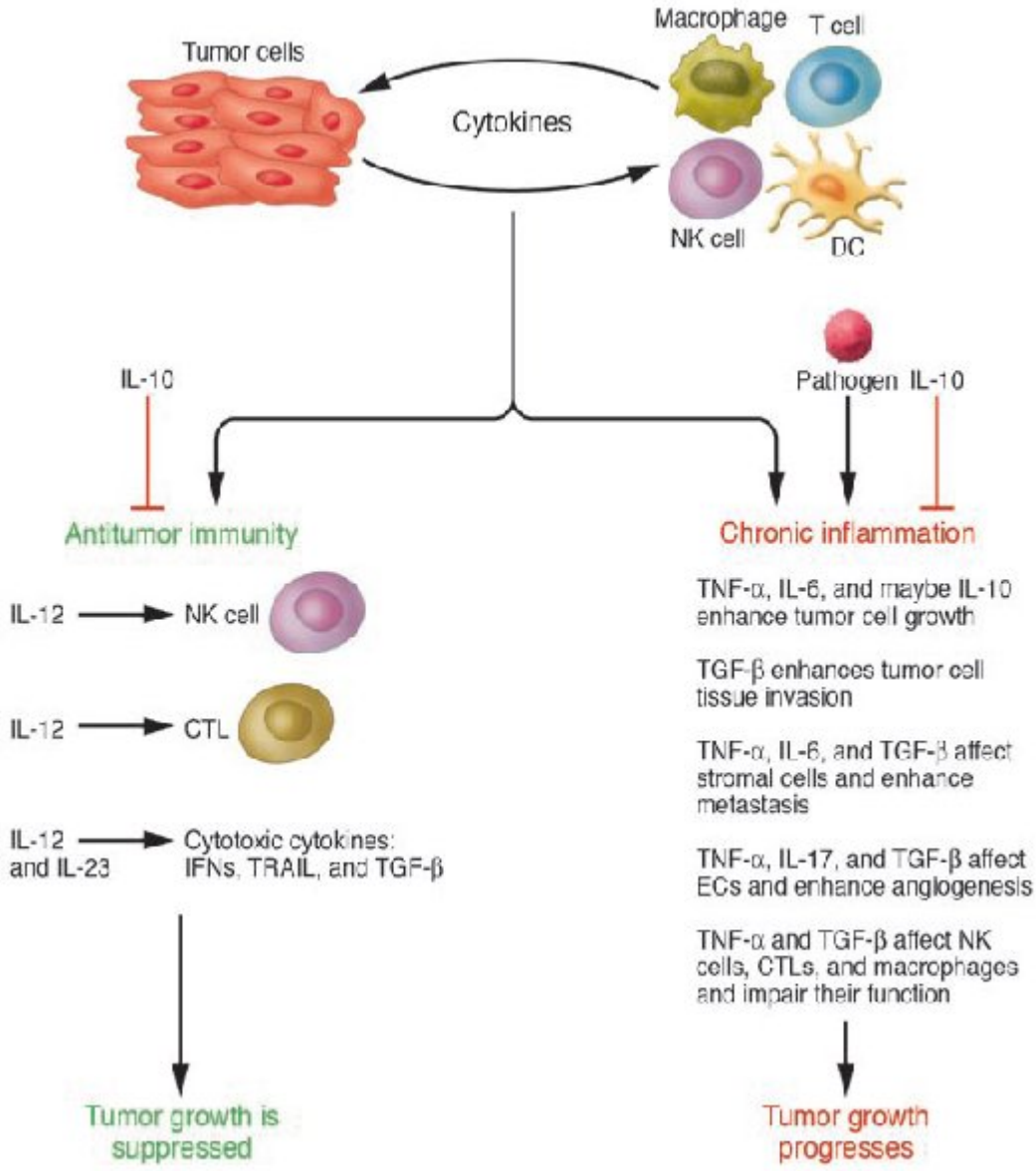

Figure 1.6: Representation of two outcomes of interactions between tumor cells and infiltrating inflammatory and/or immune cells in the tumor microenvironment.
Cytokines secreted by tumor and inflammatory/immune cells can either promote tumor development and tumor cell survival or exert antitumor effects. Chronic inflammation develops through the action of various inflammatory mediators, including TNF-α, IL-6, and IL-17, leading to eradication of antitumor immunity and accelerated tumor progression. However, TRAIL, through direct induction of tumor cell apoptosis, IL-10, through antiinflammatory effects, and IL-12, through activation of CTLs and NK cells and expression of cytotoxic mediators, can lead to tumor suppression. The multipleactions of TGF-β (cytotoxic in colon cancer cells, and having both positive and negative effects on the tumor microenvironment) and IL-23 explain their dual roles in tumor development. (Lin and Karin, 2007).

precursor is inactive; IL-1β is mainly active as an intracellular precursor (31 kDa) or as a membrane-associated form (23 kDa), but is only marginally active as a secreted 17.5 kDa molecule. Mononuclear cells manifest the strongest secretory capacity of IL-1α and IL-1β, whereas diverse nonphagocytic cells generally secrete low levels of IL-1β. IL-1α is only rarely secreted by living cells, except for activated macrophages, and in contrast to IL-1β, IL-1α is not commonly detected in blood or in body fluids, except during severe disease, in which case the cytokine may be released from dying cells. Diverse effects of the IL-1 molecules on tumor development have been described (Apte and Voronov, 2002). On the one hand, antitumor effects of IL-1 have been described in experimental tumor systems, mainly due to its ability to costimulate T cell activation, to induce cytokine secretion in specific as well as nonadaptive immune cells, and to potentiate the differentiation and function of immune surveillance cells. On the other hand, IL-1 potentiates invasiveness and metastasis of malignant cells, mainly by inducing adhesion molecule expression on the tumor cells as well as on endothelial cells (Apte and Voronov, 2002; Dinarello, 1996). In addition, IL-1 may stimulate the production of invasiveness-promoting factors such as matrix metalloproteinases, growth factors, or angiogenic factors by the malignant cells or by cellular elements in the tumor's microenvironment. The diverse effects of the IL-1 molecules on malignant processes have hindered the use of IL-1 as an antitumor agent in clinical trials (Apte and Voronov, 2002).

1.8 Toll like Receptors (TLRs)

TLRs are best-known for their ability to recognize conserved microbial structures that were originally named PAMPs (pathogen-associated molecular patterns) by Janeway (1989). Despite their name, PAMPs are common to all microorganisms regardless of their pathogenicity. The best-characterized TLR microbial ligands are as follows: lipopolysaccharide (LPS; endotoxin) from Gram-negative bacteria, which stimulates TLR4; bacterial lipoproteins and lipotechoic acid and fungal zymosan, which stimulate TLR1, TLR2 and TLR6; bacterial flagellin, which activates TLR5; a profilin-like molecule from the protozoan *Toxoplasma gondii*, which activates TLR11; unmethylated CpG motifs present in DNA that function as stimulators of TLR9; double-stranded RNA that activates TLR3; and single stranded RNA that can stimulate TLR7 and TLR8. In addition to microbial ligands, an increasing number of endogenous ligands are being reported as candidate stimulators of TLRs, in particular of TLR2 and TLR4. These

include heat shock proteins (HSP60, HSP70, endoplasmin, HSPB8 and α-crystallin A chain) (Vabulas *et al*., 2001; 2002), high mobility group box 1 (HMGB1) (Park *et al*., 2002; 2004), uric acid crystals (Liu-Bryan, 2005), surfactant protein A (Guillot *et al*., 2002), and various products of the extracellular matrix such as fibronectin (Okamura *et al*., 2001), heparan sulphate (Johnson *et al*., 2002), biglycan (Schaefer *et al*., 2005), fibrinogen (Smiley *et al*., 2001), oligosaccharides of hyaluronan (Termeer *et al*., 2002) and hyaluronan breakdown fragments (Jiang *et al*., 2005; Taylor *et al*., 2007).

Data has indicated that TLRs (and IL-1–IL-18R signalling) have a crucial role in the development of tumours as they arise in their natural microenvironment, thus revealing a previously unknown aspect of tumorigenesis. It has been suggested that the response of stromal cells such as tissue-resident macrophages to the death of hepatocytes is crucial to the proliferation and expansion of pre-cancerous cells and tumour promotion (Maeda *et al*., 2005). This promotion is the result of the NF-κB-dependent production of inflammatory mediators such as IL-6 following recognition of necrotic hepatocytes by tumour stroma (Maeda *et al*., 2005; Naugler *et al*., 2007). These studies indicate that TLR signalling contributes to the growth of tumours in numerous organs and thus may represent a general principle of tumorigenesis. whether TLRs are involved in tumour initiation is not yet clear. A formal role of TLRs in initiation with concatenate inflammation is yet to be determined; however, one can envision several possible roles for TLRs in initiation. TLR signalling has been shown to augment tumour cell adhesion and invasion and increase vascular permeability (Wang, 2003), although a role for TLRs in the natural events of metastasis has yet to be determined, nonetheless, harnessing TLRs for cancer immunotherapy and vaccines is promising.

1.9 Lung cancer

Lung cancer is a major health problem worldwide. The incidence is increasing globally at a rate of 0.5% per year. It is the leading cause of cancer mortality in most of the countries in the world (Jemal *et al*., 2002; Magarth and Litak, 1993). It remains the most lethal form of cancer in men and has now surpassed breast cancer in women as well in USA, where 170,000 new cases are diagnosed per year (Jemal *et al*., 2002). The worldwide incidence is 14% whereas it constitutes 6.8% of all cancers in India (Nanda Kumar, 2001). It is the leading cancer of both sexes in three of the Urban Cancer Registries (Bhopal, Delhi and Mumbai) in India (Nanda Kumar, 2001). In Kashmir it

ranks second among all cases in males. Non-small cell lung cancer accounts for nearly 85% and small cell lung cancer accounts for 15% to 20% of cases. Despite advances in imaging techniques and treatment modalities, the prognosis of lung cancer remains poor, with a five-year survival of 14% in early stages and less than 5% in locally advanced stages (Mghfoor and Michael, 2005; Montain, 1986). Unfortunately only 20-30% of patients present with an operable disease, while most of the patients present in an advanced stage II and III (Overholt *et al*., 1975). Evidently there is urgent need to understand the mechanistic details of lung cancer pathogenesis and devise strategies for its effective prevention. Evaluating immune interplay in lung tumorigenesis is an untreaded research area and as such holds great promise in unravelling therapies for lung cancer in particular and other carcinoma in general.

Tumor escape from the host is facilitated by the ability of human tumors to actively subvert antitumor immunity by downregulating or completely suppressing local and systemic innate as well as adaptive antitumor immunity by a variety of mechanisms. Several lines of evidence indicate that inflammatory cells and cytokines found in tumors are more likely to contribute to cancer progression rather than to mount an effective host anti-tumor response. Tumor-Infiltrating Macrophages (TIMs) are known to constitute a large part of tumors especially carcinomas (tumors of epithelial origin) and it is established that these TIMs are recruited from the circulating monocyte pool. The macrophages once in the tumor vicinity are 're-educated' for a phenotype that is beneficial for tumor growth/progression/metastasis, which forms a part of a broader concept of Immunoediting. The tumor favoring phenotype is brought about by the crosstalk through the microenvironment of the tumor cells and is mainly determined by the cytokine/chemokine milieu i.e. combination of pro-inflammatory and anti-inflammatory cytokines. Some cytokines like TNF, IL-6, IL-10, IL-12, IL-1, IL-8, TGF-β are predominantly seen to be involved in generated such a milieu. The regulation of these cytokines skews the macrophage phenotype from Classical 'M1' to somewhat suppressed 'M2', also called TAM (Tumor-Associated Macrophage) phenotype. Another important consequence of such cytokine milieu is suppression/regulation anti-tumor responses from Cytotoxic (NK & T) cells. This suppression is evaluated by studying the expression of cytokines specific to these cells like IL-2, IFN-γ, IL-10. Thus, TAMs are believed to be key orchestrators of cancer- related inflammation, and the neoplastic cells are thought to actively guide monocyte recruitment from blood into tumor tissues to their own advantage.

It becomes imperative and interesting to understand inflammatory mechanisms influencing the tumor microenvironment, in turn enabling to deduce the differences between inflammation that drives cancer progression and inflammation that inhibits tumor growth. Evaluation of the interaction between tumor cells with the immune cells especially the precursor cells like that of myeloid origin would certainly shed fresh insights on tumor development and immune evasion. Here, we try to evaluate the cytokine expression when immune cells are present in the immediate vicinity of the carcinoma cells especially lung carcinoma cells of human origin.

2.1 Materials:

2.1.1 Chemicals and Reagents

3, 3'-diaminobenzidine tetrahydrochloride (DAB), 3, 3', 5, 5'-Tetramethyl benzidine (TMB), phorbol 12-myristate 13-acetate (PMA), propidium iodide (PI), bovine serum albumin (BSA), and proteinase K were procured from Sigma Chemicals Co. (St. Louis, MO). Ficoll-Paque Plus^{TM} reagents were purchased from Amersham Biosciences (Amersham, Piscataway, NJ). Src-kinase inhibitor Genistein, MAPK (MEK-1) inhibitor PD98059, p38 inhibitor SB202190, PI3 kinase inhibitor Ly294002, JNK inhibitor JNKII, NF-κB inhibitors PDTC, Whortmanin were obtained from Calbiochem (Darmstadt, Germany) and/or Cell Signaling Technology Inc. (Dancers, MA). EGFR (tyrosine kinase) inhibitor Tyrphostin AG1478, and Farnesyl Thiosalicylic acid (FTS) was procured from Caymen Chemicals Inc (USA). RPMI-1640, DMEM and fetal calf serum (FCS) were obtained from Biological Industries (Kibbutz, Israel). BMEM was obtained from Lonza Inc. (USA). Mouse TNF, Mouse IL-6, Human TNF-α, Human IL-6, Human IL-10, Human IL-12p40, Human IL-12p70, Human IL-1β detection kits were obtained from BD Biosciences (San Jose, CA) and eBiosciences Ltd. (San Diego, CA);. Micro BCA^{TM} (bicinchoninic acid) protein assay kit was procured from Pierce (Rockford, IL). Nitrocellulose membranes for immuno-blotting were obtained from Advanced Microdevices Pvt. Ltd. (Ambala, India). Collagen and Fibronectin were procured from Sigma Chemical Co. (USA). The reagents used in electrophoresis and immuno-blotting were purchased from Sigma Chemical Co. (St. Louis, MO). Chemicals used in the preparation of buffers and other solutions were of analytical grade, and unless otherwise stated were obtained from E. Merck Ltd. (Mumbai, India).

2.1.2 Cell lines

The human leukemic T cell line Jurkat, human monocytic cell line THP-1 were all obtained from American Type Culture Collection (ATCC) (Manassas, VA). The Human Adenocarcinoma Cell Line, A549, was a kind gift from Dr. Devinder Sehgal, National Institute of Immunology, New Delhi, India. Human undifferentiated cell line, ChaGoK-1, Human Colon Adenocarcinoma cell line, Caco-2, Human cervical cancer cell line, HeLa, Human T-cell line, Jurkat and Mouse macrophage cell line, RAW264.7, were kindly

provided by Dr. Ayub Qadri, National Institute of Immunology, New Delhi. Mouse Lung carcinoma cell line, LL/2 (Lewis Lung carcinoma) was a kind gift from Dr. Rahul Pal, National Institute of Immunology. Human lung SV-40 transformed cell line, BEAS-2B was kindly provided by Dr. Balaram Ghosh Laboratory, Institute of Genomics and Integrative Biology, New Delhi, India.

2.1.3 Antibodies

TLR-2 antibody Monoclonal Anti-Human/Mouse CD282 TLR-2 purified Ab (T2.5 clone) was obtained from eBioscience (San Diego, CA). Human anti-CD3 antibody OKT3 were purified from culture supernatants (Hybridoma Laboratory, NII, New Delhi). Human anti-CD28 antibodies were obtained from eBiosciences Ltd. (San Diego, CA).

2.2 Preparation of buffers and other reagents

2.2.1 Phosphate-buffered saline (50mM phosphate, 150mM NaCl, pH 7.4)

Na_2HPO_4	40.5mM
$NaH_2PO_4.2H_2O$	9.49mM
NaCl	150mM

2.2.2 PBS-Tween: Tween-20 was added to PBS to a final concentration of 0.05%.

2.2.3 Tris-buffered saline (Tris base, NaCl, pH 7.6)

For 10X

$C_4H_{11}NO_3$ (Trizma Base)	24.2 g
NaCl	80 g

Adjust pH to 7.6 with conc. HCl.

2.2.4 TBS-Tween: Tween 20 was added to TBS to a final concentration of 0.05%.

2.2.5 Protein Extraction buffer (SDS lysis buffer, pH 7.5)

Tris base	20mM
EDTA	1mM
SDS	2%

2.2.6 Acetic acid-NaCl solution, pH 3.0

Acetic acid	0.1M
NaCl	0.15M

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

The solutions were prepared according to the following recipe:

2.2.7.1 Resolving gel (for 10ml) 12.0%

Acrylamide 30%, bis-acrylamide 0.8%	4.0ml (3.35ml for 10%)
Tris HCl buffer (1.5M Trizma base), pH 8.9	2.5ml
H_2O	3.35ml (4.0 ml for 10%)
SDS 10%	100µl
APS 10%	50µl
TEMED	8µl

2.2.7.2 Stacking gel (for 5 ml)

Acrylamide 30%, bis-acrylamide 0.8%	0.65ml
Tris-HCl buffer (1M Trizma base), pH 6.8	0.65ml
H_2O	3.65ml

SDS 10%	50µl
APS 10%	25µl
TEMED	6µl

SDS-Sodium dodecyl sulphate; APS-Ammonium persulphate; TEMED-N,N,N'N'-Tetramethylethylenediamine.

2.2.7.3 Laemmli sample buffer (non-reducing)

Tris-HCl buffer, pH 6.8	0.16M
SDS	2.3%
Glycerol	10%
Bromophenol blue	0.1%

2.2.7.4 Electrode buffer

Glycine	192mM
Trizma base	25mM
SDS	3.5mM

2.2.7.5 Staining solution

Coomassie brilliant blue	0.25%
Methanol	40%
Glacial acetic acid	10%

2.2.7.6 Destaining solution

Methanol	40%
Glacial acetic acid	10%

2.2.8 Western Blot

The reagents used in Western blotting were as follows:

2.2.8.1 Transfer buffer

Glycine	192mM
Trizma base	25mM
Methanol	20%

2.2.8.2 Ponceau-S (10X)

Ponceau S	26.3mM
Sulphosalicyclic acid	1.18M
Trichloroacetic acid	1.84M

2.2.8.3 Substrate for Western blot

0.05mg 3, 3'-diaminobenzidine was dissolved in 1ml PBS and 1µl of 30% H_2O_2 was added. Substrate was prepared fresh.

2.2.9 Buffers for ELISA:

2.2.9.1 Carbonate buffer, pH 9.5

Na2CO3 32 mM

NaHCO3 74 mM

2.2.9.2 Citrate phosphate buffer, pH 5.6

Citric acid 22.1 mM

Na2HPO4 51.4 mM

2.2.10 Substrates for ELISA

2.2.10.1 TMB-TBABH solution

3, 3', 5, 5'-Tetramethyl benzidine (TMB)	41mM
Tetramethylammonium borohydride (TBABH)	8.2mM
N,N-Dimethylacetamide (DMA)	10ml

The solution was stored in an airtight dark glass container at 4°C.

200µl of TMB-TBABH solution and 3µl of 30% hydrogen peroxide (H_2O_2) were added to 8ml citrate phosphate buffer. The substrate was prepared immediately before use.

2.2.10.2 Ortho-phenylene diamine (OPD)

0.5mg OPD was dissolved in 1ml citrate phosphate buffer and 1µl H_2O_2 was added to it. The substrate was prepared fresh before use.

2.3 Methods

2.3.1 Maintenance of cell lines

Cells were maintained in RPMI-1640 (for THP-1, PBMC, Caco-2, ChaGoK-1, RAW264.7) or DMEM (for A549, HeLa, LL/2) or BEBM (along with additives i.e. fibronectin, collagen for BEAS-2B) supplemented with 10% heat inactivated fetal calf serum (RPMI-10) at 37°C in a humidified CO_2 (5%) incubator. Cells were centrifuged at 315 $\times g$ for 5 min, washed twice in serum-free RPMI-1640, resuspended in RPMI-10 and grown in 75cm^2 tissue culture flasks. The cells were subcultured as per ATCC recommended guidelines.

2.3.2 Co-culture of Carcinoma (tumor) cells and immune cells

Representative Human Lung Carcinoma cells, **A549** (Well differentiated Adenocarcinoma) & **ChaGoK-1** (Undifferentiated Squamous Cell Carcinoma) were co-cultured with Human Monocytic cell line, **THP-1** (*in-vitro*) and Peripheral Blood Mononuclear Cells, **PBMCs** (*ex-vivo*) in standardized ratio of ~1 : 10 for respective cell

types.. Similar, ratios were also used for co-cultures of Human Lung Epithelial cells, **BEAS-2B** (Transformed only), Human Colon Adenocarcinoma, **Caco-2** and Human Cervical Adenocarcinoma, **Hela**, if not stated otherwise. Mouse co-culture systems between LL/2 and Raw264.7 or mixed co-cultures between Human tumor cells and Mouse macrophages or *vice versa* were also set accordingly. Tumor and Jurkat cells were used in ~1: 100 ratio.

2.3.3 Culture supernatants

Briefly, tumors cells were plated first and allowed to grow and adhere for about 24 hours. Then, the culture media was taken out, the cells washed and added with monocytes along with fresh culture medium. Culture supernatants were collected at various time points and assayed for various cytokines. Also, conditioned media from tumor cells was collected at various time points, added to monocytes and culture supernatants were assayed for cytokines.

2.3.4 Protein estimation

Protein concentrations were determined using the Micro BCA^{TM} (bicinchoninic acid) protein assay kit (Pierce, USA). The assay was performed according to the instructions provided by the manufacturer. The dilutions of the sample were made in PBS and mixed with equal volume of reagent mix (B: C: A :: 24 : 1: 25). The plate was incubated at 37°C for 1 h and absorbance was measured at 540 nm. BSA of known concentration provided with the kit was used as a standard.

2.3.4 SDS-PAGE

SDS-PAGE was carried out using the Laemmli buffer system (Laemmli, 1970). The resolving gel was polymerized in a Hoefer or BioRad Protean-3 mini gel apparatus for 30-45 min. The thickness of the gel was 1.5mm. The stacking gel prepared afresh was layered on top of the resolving gel and allowed to polymerize for 15-20 min. Samples to be analyzed were mixed with Laemmli sample buffer and placed in a heating block at 100°C for 5 min before loading into wells. Electrophoresis was carried out at a constant current of 30mA.

2.3.5 Western Blot

Western blot was carried out by the method described by Towbin *et al.* (1979). The sample to be analyzed separated in a 12% SDS-PAG and transferred to a nitrocellulose (NC) membrane (MDI, India) at a constant current of 300mA for 2 h using a Bio-Rad transfer apparatus (BioRad, USA). The transfer of proteins was ascertained by staining the NC membrane with Ponceau-S (1X). The membrane was blocked for 1 h at room temperature with 1% non-fat milk protein prepared in PBS and subsequently probed with the appropriate primary antibody, followed by HRP-labeled secondary antibody and developed using Enhanced Chemiluminescence reagents.

2.3.6 Human TNF-α, Human IL-6, Human IL-10, Human IL-12p40, Human IL-12p70, Human IL-1β, Human IL-8, Human IFN-γ, Mouse TNF, Mouse IL-6 ELISA

The assay was carried out according to the instructions provided by the manufacturer with slight modifications. Briefly, a 96-well microplate (Maxisorp, Nunc) was coated overnight at 4°C with 50µl capture antibody (diluted 1: 250 in 100mM carbonate buffer, pH 9.5 or as provided in manufacturers-BD Biosciences instructions). The plate was washed 3 times with PBS-Tween (PBST) and blocked with PBS-BSA-1% (200µl/well) for 1 h at 37°C. After washing, samples were added to each well and the plate was incubated for 1 h at 37°C. Subsequently, the plate was washed and incubated with detection reagent mix (detection antibody + avidin-HRP) diluted 1: 250 in PBS-BSA 1%. After 1 h incubation, the plate was washed and the enzyme activity determined by adding freshly prepared substrate solution containing TMB/TBABH/H_2O_2 (75µl/well). The reaction was stopped with 125µl of 2N H_2SO_4 and the absorbance was read at 450nm (or as advised in manufacturer's instructions.

2.3.7 Generation of Macrophage precursors and T cell blasts from human PBMCs

Blood was collected in heparin-coated vacutainers, by venipuncture from healthy human volunteers. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque density gradient centrifugation. Briefly, fresh heparinized blood was diluted with an equal volume of PBS and slowly layered over Ficoll-Paque solution in 15ml poly-propylene tubes. 3ml of Ficoll-Hypaque was used per 10ml of blood/PBS mixture. The tubes were centrifuged at 2000 × g for 30 min at 20°C. The upper layer containing the

plasma and most platelets was removed and the mononuclear cell layer at the plasma/Ficoll-Paque interface was collected in a separate tube (Strober W, 2001). For Macrophage precursors, these cells were plated (24 well or 6 well or 25-cm^2 flask) for 12 hours. The adhering cells were analysed as macrophage precursors. The non-adherent cell were further analysed for cell blast generation. For T-cell blast generation, these non-adherent cells were washed with RPMI-1640, resuspended in RPMI-10 containing 2μg/ml of anti-CD28 antibody. Cells were then transferred to a 25-cm^2 flask (or in plates) coated with 10μg/ml of anti-CD3 antibody, and incubated for 48 h at 37°C. Dead cells were removed by Ficoll-Paque density gradient centrifugation and live cells were used to study effect of cell-free extracts .

2.3.8 Wound Healing Assay

Wound Healing Assay is used to study the effects of a variety of experimental conditions on cell migration and proliferation. Briefly, cells were grown in DMEM/RPMI supplemented with 10% FBS. Cells were seeded into 24-well or 6 well tissue culture plates in a density that, after 24 hours of growth, they should reach ~70-80% confluence as a monolayer. Gently and slowly a scratch (wound) was made on the monolayer with a new 1 ml pipette tip across the centre of the well. While scratching across the surface of the well, the long-axial of the tip was kept perpendicular to the bottom of the well. Scratch a straight line in one direction. After scratching, the wells were gently washed twice with medium to remove the detached cells. The well/s was replenished with fresh medium. (Medium may contain ingredients of interest, e.g., components that inhibit/promote cell motility and/or proliferation.) Cells were grown for additional 48 hours (or the time required). Cells were washed twice with 1x PBS, then fixed with 3.7% paraformaldehye for 30 minutes/ or directly visualised and photographed. Same configurations of the microscope were maintained while taking pictures for different views of the stained monolayer or monolayer of comparing wells.

2.3.9 Propidium Iodide (PI) Staining

Propidium iodide (PI) is a membrane impermeant dye that is generally excluded from viable cells. It binds to double stranded DNA by intercalating between base pairs. PI is excited at 488 nm and, with a relatively large Stokes shift, emits at a maximum wavelength of 617 nm. Because of these spectral characteristics, PI can be used in

combination with other fluorochromes excited at 488 nm such as fluorescein isothiocyanate (FITC) and phycoerythrin (PE). Cells were harvested and aliquoted up to 1 x 10^6 cells/100 µL into FACS tubes. Cells washed 2 times by adding 2 mL of PBS, centrifuged at 300 x g for 5 minutes, and then decanted for buffer from the pelleted cells. Cells were re-suspended in 100 µL of Flow Cytometry Staining Buffer. To adjust flow cytometer settings for PI, 5 - 10 µL of PI staining solution was added to a control tube of otherwise unstained cells followed by gentle mixing and incubation for 1 minute in the dark. PI fluorescence (using the FL-2) was determined with a BD FACS caliber™ instrument. Data was acquired for unstained cells and single-color positive controls. 5 - 10 µL of PI staining solution was added to each sample just prior to analysis. The stop count was set on the viable cells from a dot-plot of forward scatter versus PI.

2.3.10 Inhibitor Assay

Tumor cells were treated with various signaling inhibitors for two (2) hours after overnight plating. Cells were washed 3-5 times with serum free culture medium to wash off the inhibitors. Subsequently, tumor cells were co cultured (as shown in section 2.3.2) and assayed for cytokines in the culture supernatants.

2.3.11 Concentration of Inhibitors

The viable inhibitor concentrations were first standardized for each tumor cell type based on their IC_{50} scores. Subsequently, the concentrations used were

MAPK (MEK-1) inhibitor, PD98059	200µM
p38 inhibitor, SB202190	50µM
PI3 kinase inhibitor, Ly294002	10µM
JNK inhibitor, JNKII	50µM
NF-κB inhibitor, PDTC	200µM
Whortmanin	10nM

DMSO was the vehicle (solvent) for inhibitors.

2.3.12 TLR-2 Blocking Assay

Tumor cells were incubated with TLR-2 blocking antibody (Monoclonal Anti-Human/Mouse CD282 TLR-2 purified Ab) alongwith its Isotype (IgG_1) control for two (2) hours at room temperature after overnight plating. Cells were washed 3-5 times with serum free culture medium to wash off any free antibody. Subsequently, tumor cells were co cultured (as shown in section 2.3.2) and immune-assayed for cytokines in the co-culture supernatants.

2.3.13 Statistical Analysis

Statistical analysis was done using Graph Pad Prism (Ver. 5.0) software. For comparative studies, data were analyzed by One Way ANOVA with multiple comparisons using Dunnett's test. Data were represented as Mean ± SD and values were considered statistical significant for $p < 0.05$ (CI = 95%) from atleast three (or five) independent experiments or otherwise mentioned.

3.1 Tumor cells induce regulated expression of Tumor Necrosis Factor (TNF-α) from monocytes

3.11 A549 cells trigger Tumor Necrosis Factor-α from Monocytes

We used a co-culture system, which mimics the actual microenvironment scenario, to study the interaction between immune cells especially monocytes with tumor cells. TNF-α is a well known pro-inflammatory cytokine and secreted upfront in most immune responses. When Human Lung Adenocarcinoma cells, A549 were co-cultured with Human Monocytes, THP-1, TNF-α was detected in the culture supernatants from these co-cultures. Next, we co-cultured A549 cells with THP-1 cells at various cell numbers to standardise the cell ratios for optimal responses. A549 and THP-1 cells did not express TNF-α by themselves. Tumor cell numbers ranging from 10^3 to 2×10^5 were co-cultured with 2×10^5 THP-1 cells in 24 well cell culture plates. It is observed that the ratio of 1: 10 of tumor and monocytes cells, respectively, gave optimal TNF-α response (Figure 3.1). Incidentally, tumor cells and myeloid cells in similar ratios are seen intervasating solid tumors or carcinoma. For the following co-culture experiments we maintained such ratios.

3.12 Tumor Necrosis Factor-α (TNF-α) expression is early and robust from in all co-culture systems

To check whether the expression of TNF-α was specific to A549 cells or the expression could be corroborated with other co-culture systems with tumor cells of different origins we co-cultured representative tumor cells of colon, Caco-2 and cervix, HeLa with THP-1 cells at already mentioned ratios i.e. ~ 1:10. We immunoassayed for TNF-α expression and deduced that all model tumor cells when co-cultured with THP-1 cells gave robust TNF responses (Figure 3.2). Following the kinetics of TNF-α expression in the co-culture supernatants it is observed that TNF is detectable as early as 1 hour post co-culture. The response peaks around 4-6 hour time point and recedes thereafter. It is noteworthy that a potent TNF-α concentration of ~1000 pg/ml is achieved in all co-cultures, indicating to a definitive role during the interaction of these two cell types. The

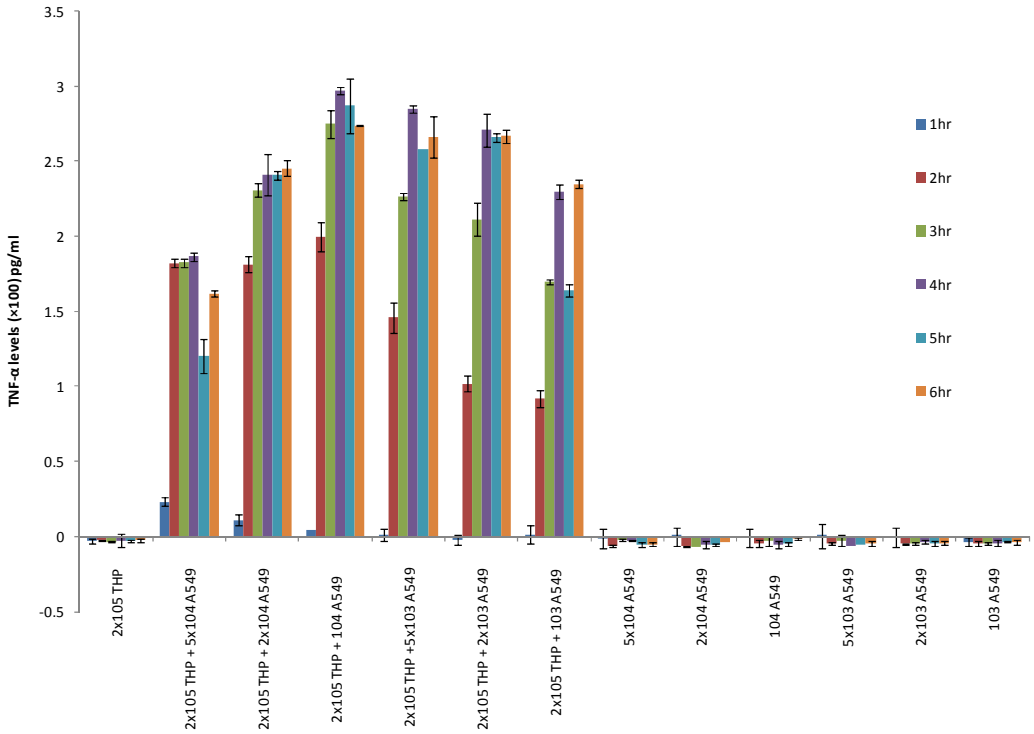

Figure 3.1: TNF expression in culture supernatants of A549 and THP-1 at denoted cell numbers. *Data were represented as Mean Concentration of TNF-α ± SD. Values were considered as statistically significant for $p < 0.05$ from at least five independent experiments.*

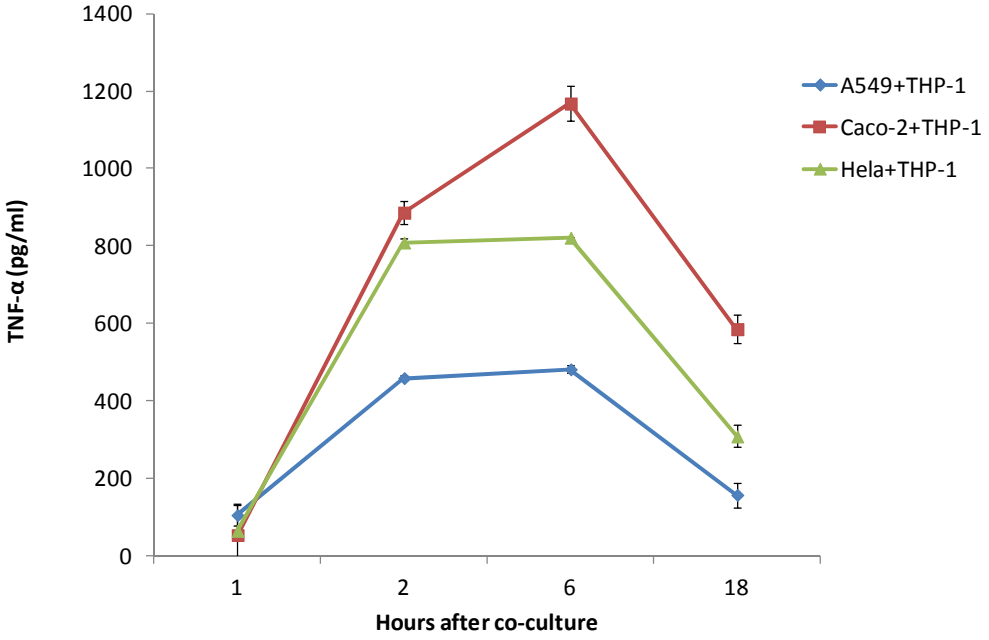

Figure 3.2: TNF-α expression kinetics in co-cultures of various Carcinoma (5 x 10^4) + THP-1 (2 x10^5) cells. *(Data were represented as Mean Concentration of TNF-α ± SD. Values were considered as statistically significant for $p < 0.05$ from atleast five independent experiments.*

receding levels of TNF-α also underline this argument. The tumor cell number affects the potency but not the kinetics of the response, probably owing to concentration of the stimulus from tumor cells. The response is faster and as robust, if not more, than the known ligands of TNF activation from THP-1 cells like Flagellin, LPS and Pam3CSK4 (from previous experiments-data not shown).

3.13 Conditioned medium (C.M.) from tumor cells also initiates Tumor Necrosis Factor (TNF-α) expression in monocytes but with different kinetics

To validate the response from human PBMCs in co-culture systems, 2×10^4 A549, Caco-2 and HeLa cells were co-cultured with ~ 0.25 million PBMCs obtained by Ficoll-Paque (GE Amersham) on blood from volunteers (Section 2.3.7). TNF-α expression is robust and correlates well with kinetics seen with THP-1 cell based co-cultures, presented previously. Again, the decrease of TNF at later time points emphasises the possible role of TNF. This would mean that either the already released TNF is consumed rapidly at these late time points or the expression is regulated (or switched off) after achieving a threshold.

To ascertain the nature of the stimulus that triggered the TNF-α expression, conditioned medium from tumor cells (24 hour culture medium of ~ 80% confluent cells) was incubated with monocytes. Although, in all cell-cell co-culture systems, TNF levels peaked at around 6 hours after co-culture and receded thereafter but when PBMCs were treated with Conditioned Medium from tumor cells such kinetics was altered, as the receding of TNF levels at later time points was not observed (Figure 3.3). This also holds well when THP-1 cells are treated with Conditioned Medium as well (from previous experiments-data not shown). Thus, the stimulus was not only tumor cell associated but also secretory in nature. The absence of late recession of TNF-α levels in conditioned medium treated monocytes emphasises sensing of TNF-α by tumor cells and actually regulating its expression. The fact that such expression is seen in all the co-cultures under study underlines the importance of this cytokine based interaction.

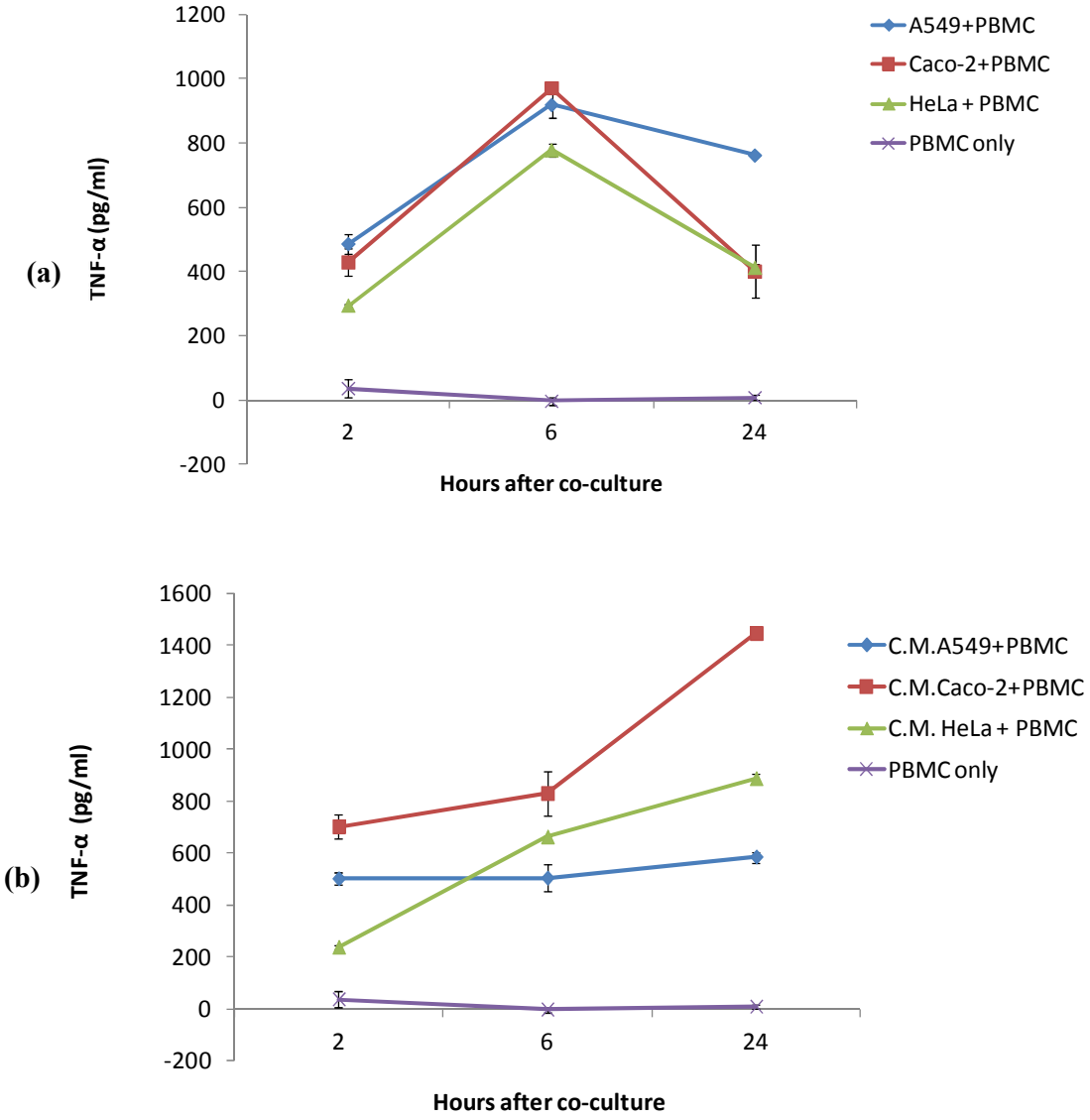

Figure 3.3: TNF-α expression in co-cultures of various

(a) Carcinoma cells ($5*10^4$) + adh. PBMCs (2.7×10^5) cells, and

(b) their Conditioned Media + adh. PBMCs (2.7×10^5) cells.

(Data were represented as Mean Concentration of TNF-α ± SD. Values were considered as statistically significant for $p < 0.05$ from atleast five independent experiments).

3.14 Comparative TNF response kinetics from Monocytes triggered by various cells of human lung origin

Cells of human lung origin A549, ChaGoK-1 and BEAS-2B were co-cultured with THP-1 at various cell numbers. The potency and universality of the effector TNF inducing ligand(s) was evaluated by screening for TNF-α responses from monocytes, which apparently is one of the first cytokines to be triggered. Comparison of the TNF expression kinetics reveals that though A549 cells are more potent than ChaGoK-1 and BEAS-2B cells, especially at lower cell numbers, all tumor cell types of the lung origin elicit TNF-α expression with typical kinetics (Figure 3.4). This further strengthens the argument that the phenomenon of cytokine induction may be common for carcinomas or solid tumors.

3.15 Murine Lung tumor cells initiate secretion of TNF from mouse macrophages

LL/2 or LLC (lewis lung carcinoma) cells, the representative murine tumor cells co-cultured with Mouse macrophages, RAW264.7 were studied for TNF release. TNF was detected in mouse co-culture supernatants as well (Figure 3.5). This suggests to similarities in TNF induction in mouse and human models.

3.16 Human Lung carcinoma cells are able to initiate TNF from Mouse Macrophages

Epithelial cells are not known to express TNF-α and as such the TNF-α expression in the co-cultures is attributed to monocytes. To validate this, mouse macrophages i.e. RAW264.7 were co-cultured with lung tumor cells of human origin i.e. A549, at already established cell ratios. Indeed, only mouse TNF was detected in co-culture supernatants, confirming that the origin of TNF was strictly from macrophages or monocytes. Again, TNF was detected as early as 1 hour post co-culture, which is strikingly similar to human co-culture system (Figure 3.6). Thus, the potency of the TNF inducing stimulus is comparable between human and murine origins. These findings also point to similarity of the stimulus for TNF induction in human and mouse systems. In other words, the cytokine triggering factors(s) from tumor cells from human and mouse carcinoma cells may be common.

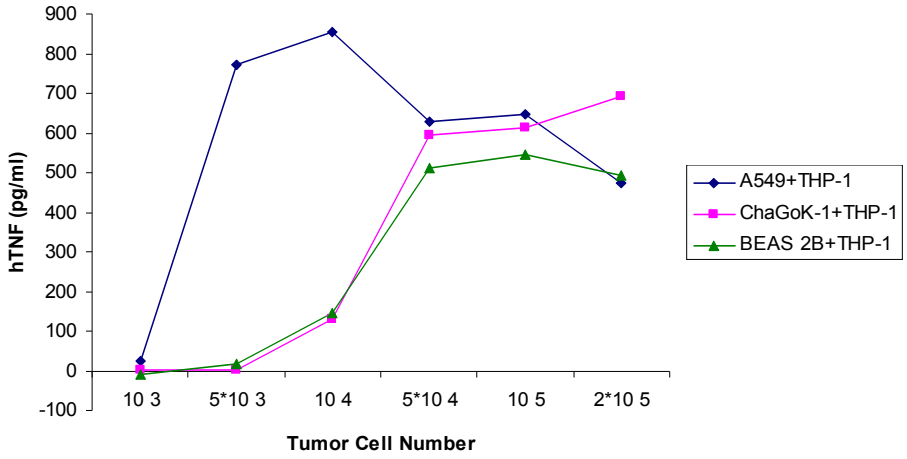

Figure 3.4: Human TNF-α (hTNF) expression in co-cultures of THP-1 with A549/ChaGoK-1/BEAS-2B cells (Comparative kinetics) at 2 hour time point. *Data were represented as Mean Concentration of TNF-α ± SD. Values were considered as statistically significant for $p < 0.05$ from atleast five independent experiments.*

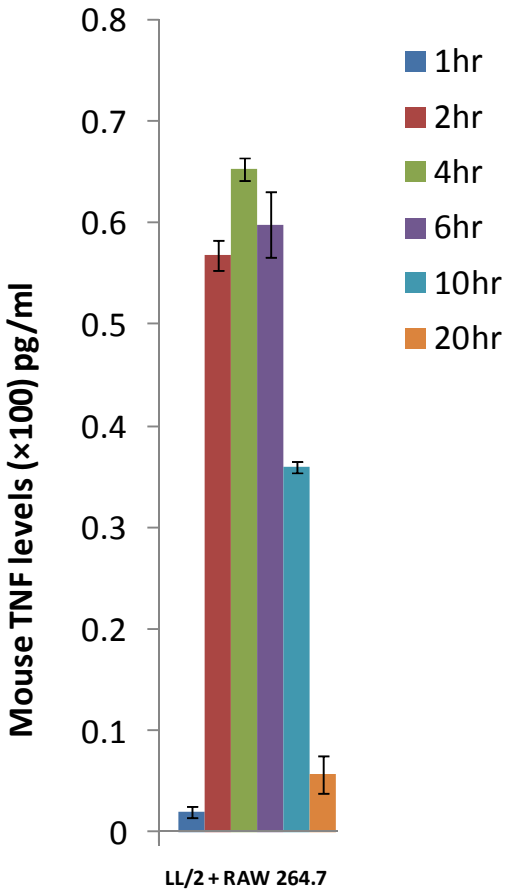

Figure 3.5: LL/2 cells induce TNF expression from RAW264.7 cells. (LL/2: 2×10^4 cells; RAW264.7: 2×10^5 cells). *Data were represented as Mean Concentration of TNF ± SD. Values were considered as statistically significant for $p < 0.05$ from atleast five independent experiments.*

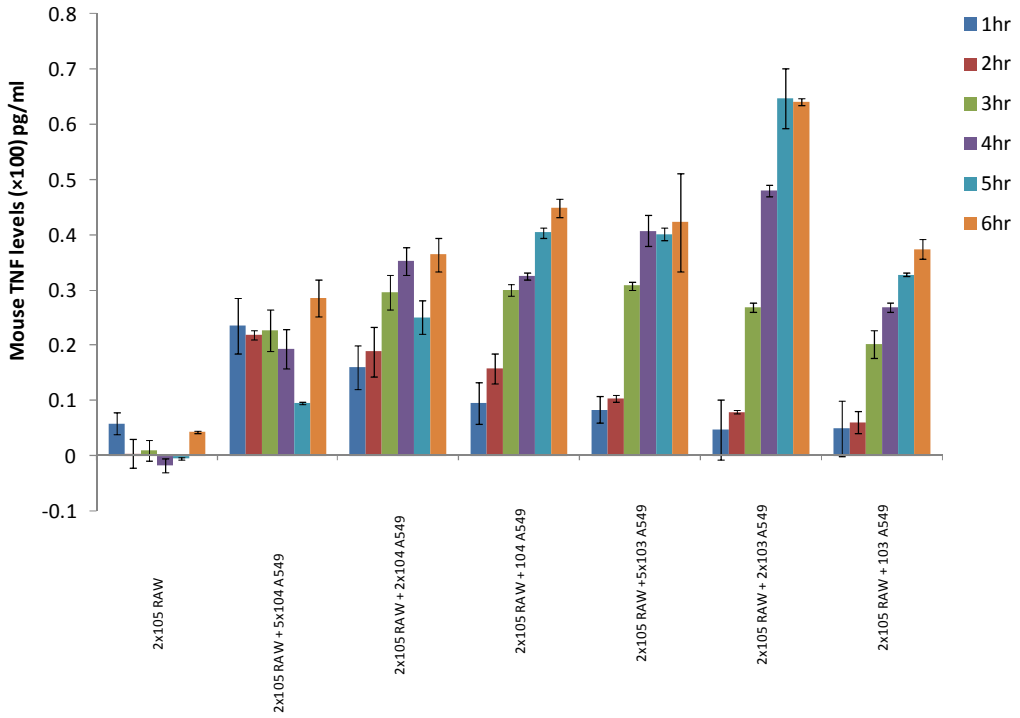

Figure 3.6: Murine TNF expression in co-cultures of A549 and RAW264.7 cells at various cell-cell ratios. *Data were represented as Mean Concentration of mTNF ± SD. Values were considered as statistically significant for $p < 0.05$ from atleast five independent experiments.*

3.17 Methanol fixed A549 cells are able to induce TNF-α from Monocytes without regulation

To gain more insights into the nature of TNF inducing stimulus from tumor cells, we compared TNF-α expression of A549 and THP-1 co-culture with methanol fixed A549 and THP-1 co-culture. Methanol fixing renders the cells dead and as such the generation and regulation of TNF inducing stimulus would also cease. Surprisingly, the TNF-α inducing capability is hardly affected but there is loss of regulation as TNF-α levels do not recede in co-cultures of methanol fixed A549 cells (Figure 3.7). It can be inferred that viable tumor cells actually sense and regulate the TNF-α levels in the co-culture. Since no fresh stimulus is synthesised by tumor cells after fixing, it is suspected that there is considerable amount of stimulus already available on the cells. The fact that the fixed A549 cells trigger much stronger TNF responses (Figure 3.7) indicates the possible release of already stocked up TNF inducing factor(s) upon fixing of tumor cells.

3.2 Co-culture of carcinoma cells and monocytes leads to strong Interleukin (IL)-6 release

3.21 Interleukin-6 expression is induced early in exorbitant amounts from THP-1 and adherent PBMCs (macrophages) by tumor cells

IL-6 from macrophages is considered as one of the major cytokines responsible in tumor establishment and progression. Immunoassays for IL-6 in co-culture supernatants of A549 and THP-1 indicated strong expression much like TNF-α. When various tumor cell numbers against monocytes were considered, again, the in vivo ratio of ~ 1:10 showed optimal IL-6 response (Figure 3.8).

IL-6 is triggered early like TNF from PBMCs and is expressed in very high concentrations in co-cultures as well as Conditioned Media (from tumor cells) treated PBMCs (Figure 3.9). A549, Hela, Caco-2 and their Conditioned Media are able to induce IL-6 expression in co-cultures, again pointing to similar nature of the stimulus and similar mechanisms of induction for all carcinoma cell types. The kinetics of IL-6 expression is comparable for all co-cultures as well as conditioned media treated PBMCs. Presence of IL-6 seems to be relevant even at later time points as no dip in expression levels is observed, instead PBMC's keep expressing more IL-6, unlike the TNF responses.

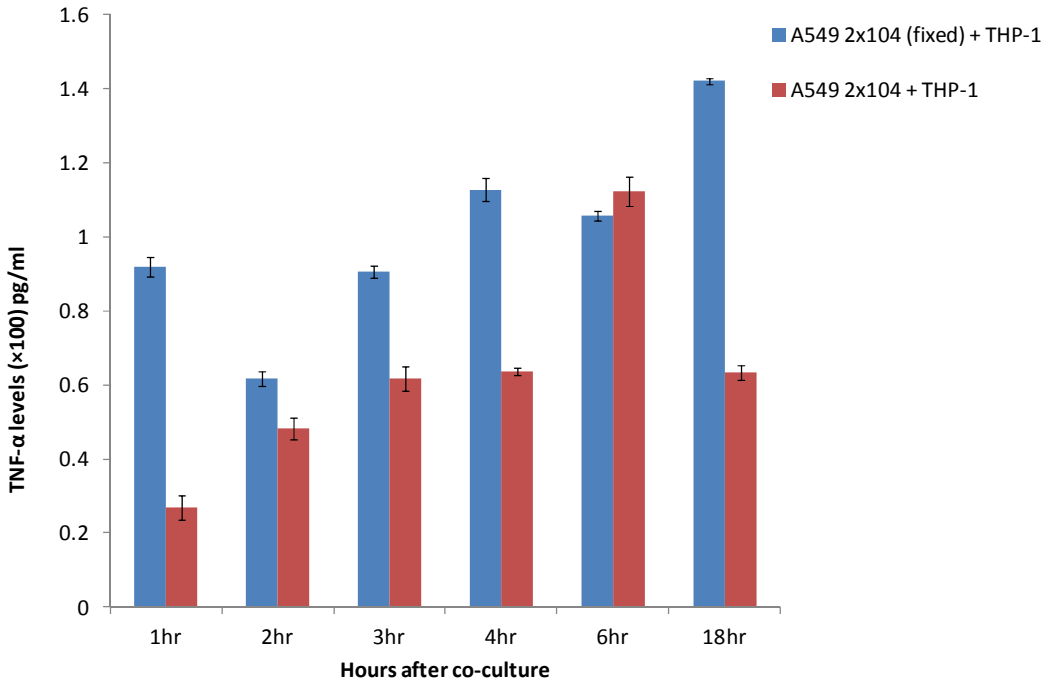

Figure 3.7: TNF-α expression in culture sups of methanol-fixed A549 + THP-1 co-cultures & A549 + THP-1 co-cultures (THP-1: $2x10^5$). *Data were represented as Mean Concentration of TNF-α ± SD. Values were considered as statistically significant for $p < 0.05$ from atleast five independent experiments.*

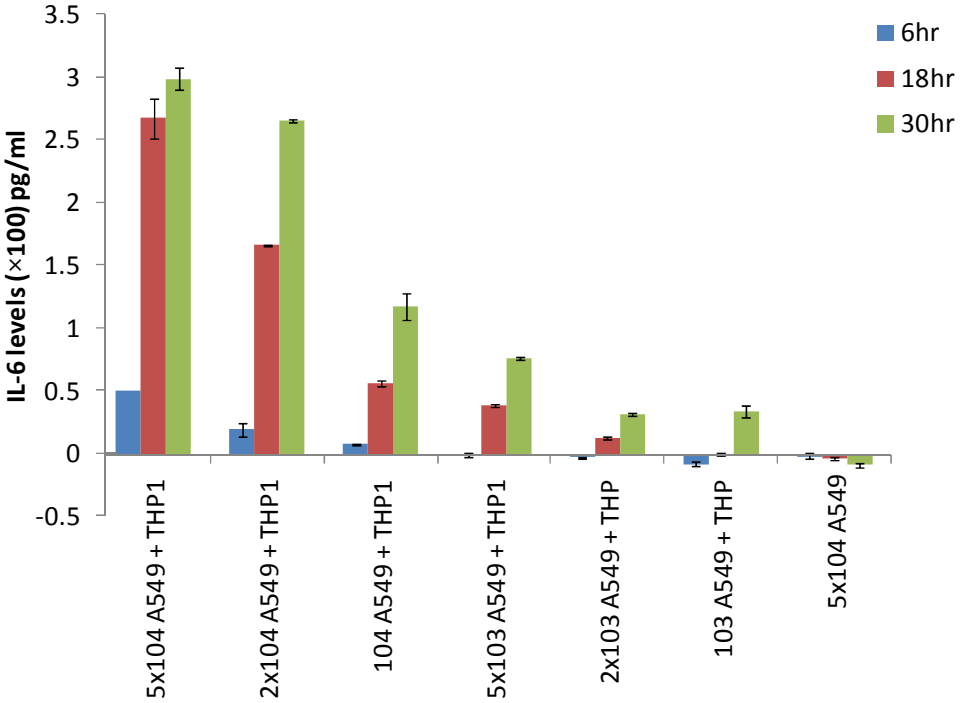

Figure 3.8: IL-6 expression in culture supernatants of A549 and THP-1 co-cultures at varied cell numbers. *Data were represented as Mean Concentration of IL-6 ± SD. Values were considered as statistically significant for $p < 0.05$ from atleast five independent experiments.*

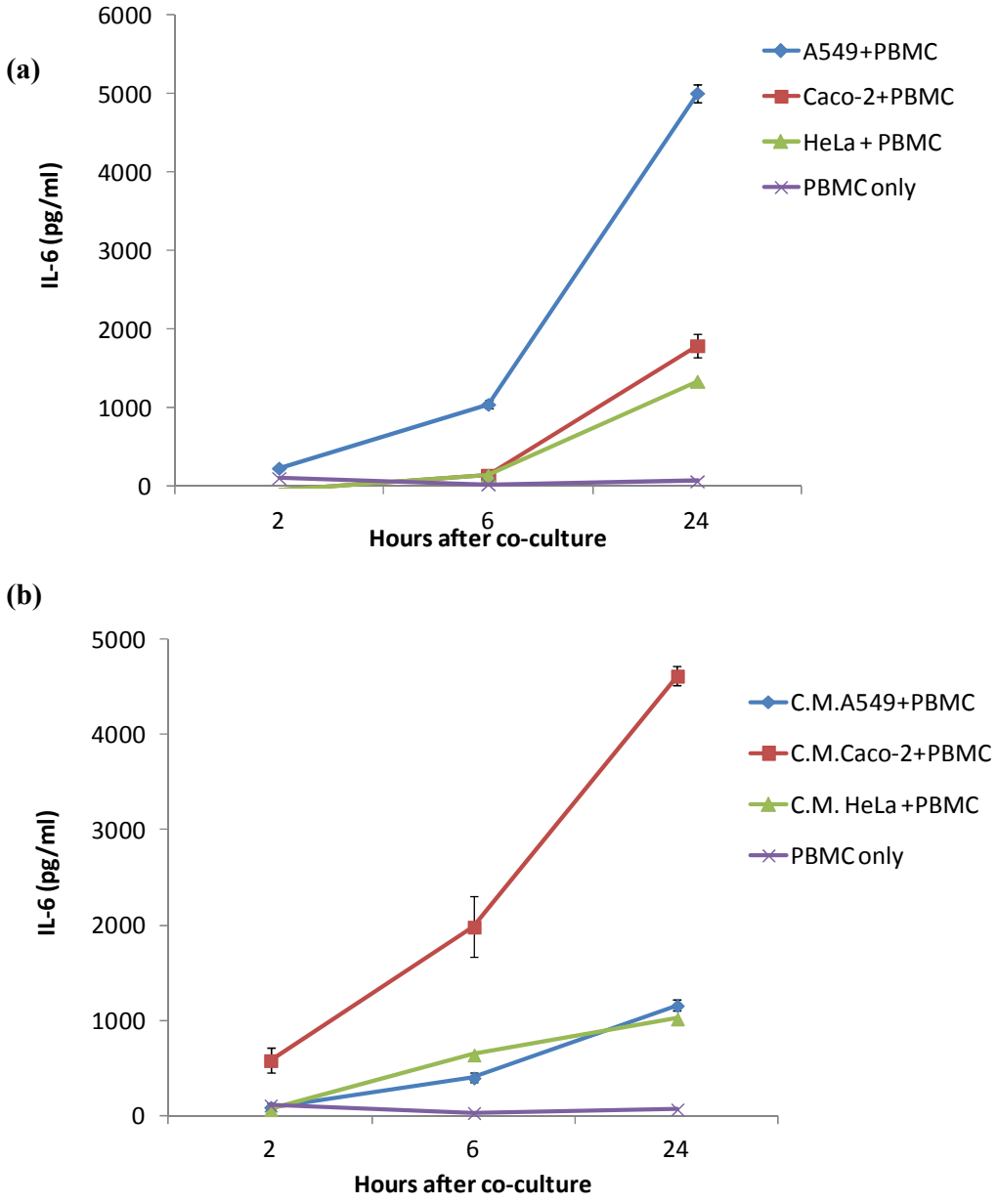

Figure 3.9: Human IL-6 expression in co-cultures of various

(a) Carcinoma cells ($5*10^4$) + adherent PBMCs ($2.7*10^5$) cells, and

(b) their Conditioned Media (C. M.) + adherent PBMCs ($2.7*10^5$) cells.

(Data were represented as Mean Concentration of TNF-α ± SD. Values were considered as statistically significant for $p < 0.05$ from atleast five independent experiments).

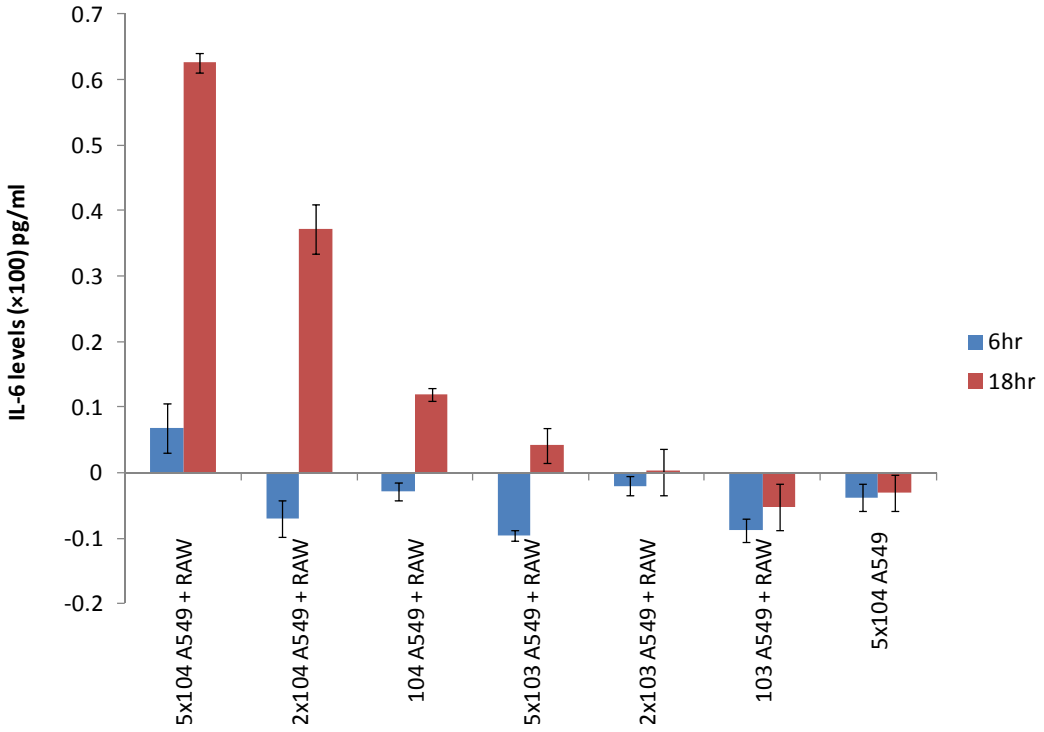

Figure 3.10: Detection of human IL-6 in culture supernatants of A549 and RAW264.7 co-cultures. *Data were represented as Mean Concentration of IL-6 ± SD. Values were considered as statistically significant for $p < 0.05$ from atleast five independent experiments.*

3.22 Human IL-6 detected in co-cultures is partially secreted by tumor cells

IL-6 apart from being secreted by almost all cells of the immune system in response to an insult, is also known to be expressed in an autocrine manner by epithelial cells. To verify this, A549 cells were co-cultured with RAW264.7 cells at various cell ratios. Significantly, human IL-6 was detected in the co-culture supernatants (Figure 3.10). This indicates that tumor cells contribute partially to the IL-6 expression in the co-culture while the rest comes from monocytes. It can also be inferred that the autocrine IL-6 is induced only after coming in contact with monocytes pointing to IL-6 inducing signal coming from monocytes. Also, the autocrine IL-6 expression, especially at late time points (Figure 3.10), could be responsible for differences in late kinetics when compared with TNF-α expression in the co-cultures.

3.3 Factor(s) from carcinoma cells also trigger IL-10, IL-12p40 from monocytes but no IL-1β or IL-12p70

3.31 Interleukin-10 is vehemently triggered from monocytes upon continued co-culture by A549 cells and A549-Conditioned Medium compared to other tumor cell types

The tumor associated alternative-M2 like phenotype of macrophages or their precursors is characterised by high IL-10 and low IL-12 expression in the microenvironment. The culture supernatants from co-cultures of A549, ChaGoK-1 and Caco-2 cells with adherent PBMCs (macrophages) and macrophages treated with Conditioned Medium from tumor cells were assayed for IL-10, an anti-inflammatory cytokine. A549 cells and its Conditioned medium were observed to be equally potent to induce high levels of IL-10 from macrophages (Figure 3.11). In comparison, ChaGoK1, Caco-2 and their respective Conditioned medium were feeble but significant inducer of such response (Figure 3.11). IL-10 was not detectable at earlier time points (not shown), suggesting that IL-10 is exerting its influence after the initial spike of TNF induction from PBMCs. This could also suggest that the cytokine milieu shifts from pro-inflammatory to anti-inflammatory phenotype. Also, the initial pro-inflammatory response could serve as a necessary trigger for the following response.

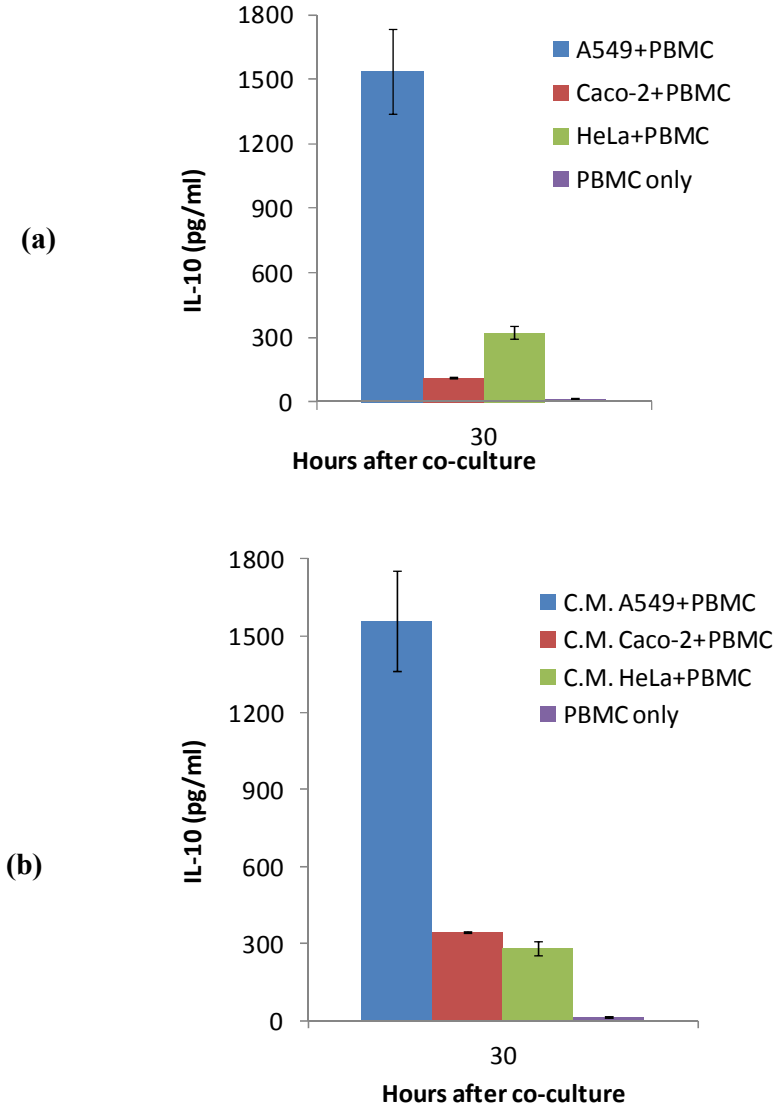

Figure 3.11: Human IL-10 in co-cultures of various

(a) Carcinoma cells ($5x10^4$) + adherent PBMCs ($2.7x10^5$) cells, and

(b) their Conditioned Media (C.M.) + adherent PBMCs ($2.7x10^5$) cells at 30 hour post coculture.

(Data were represented as Mean Concentration of IL-10 ± SD. Values were considered as statistically significant for $p < 0.05$ from atleast five independent experiments).

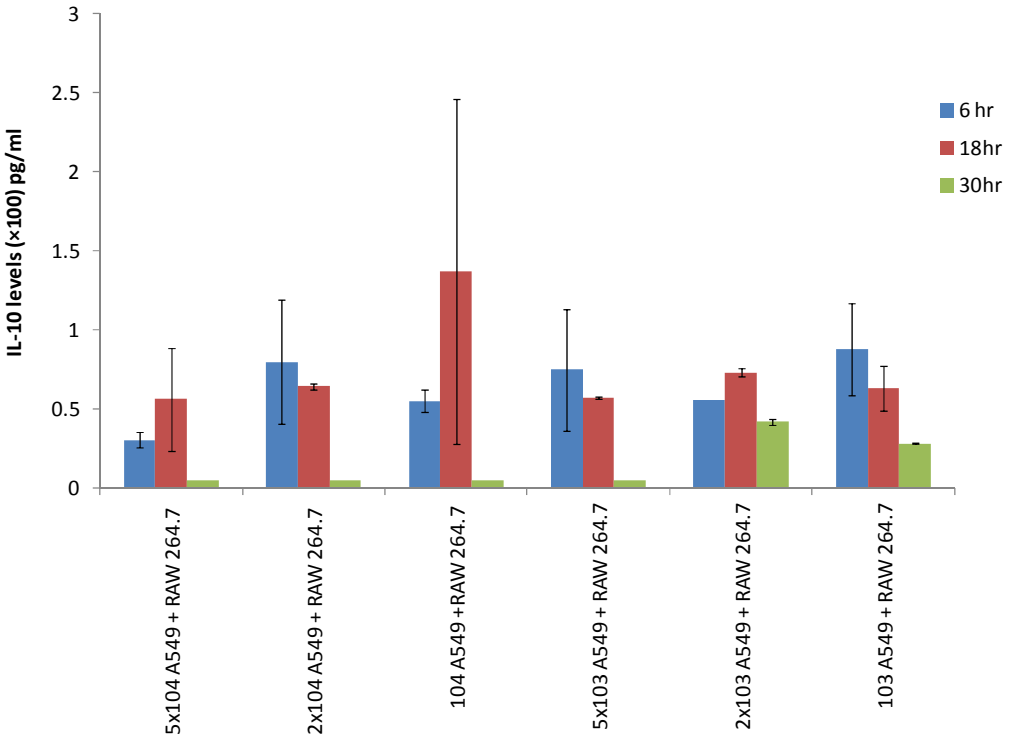

Figure 3.12: Mouse IL-10 in culture supernatants from A549+RAW264.7 co-cultures. *Data were represented as Mean Concentration of IL-10 ± SD. Values were considered as statistically significant for $p < 0.05$ from atleast three independent experiments.*

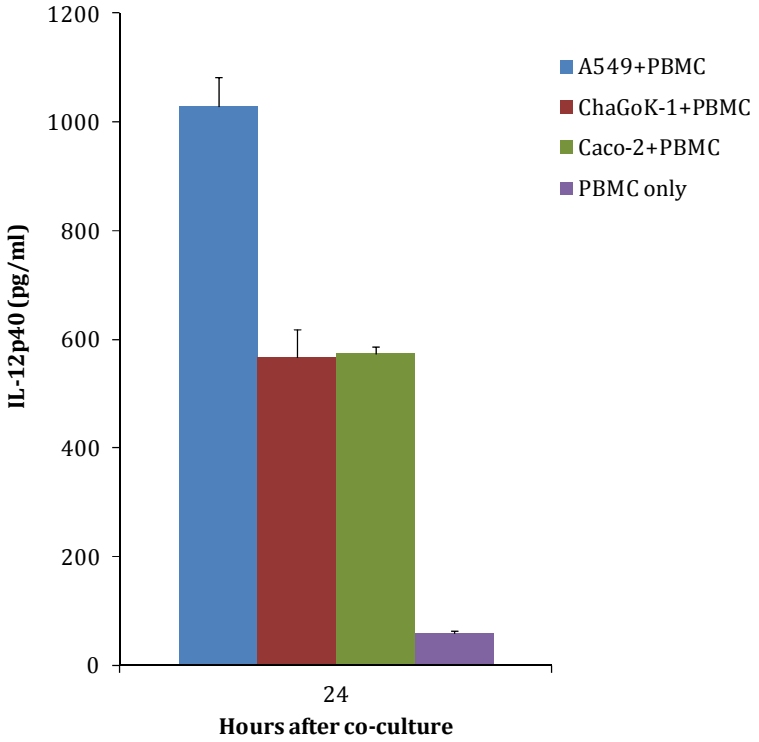

Figure 3.13: IL-12p40 expression in co-culture of A549 with adherent PBMCs. *Data were represented as Mean Concentration of IL-12p40 ± SD. Values were considered as statistically significant for $p < 0.05$ from atleast five independent experiments.*

Again to confirm the origin of IL-10 in co-culture supernatants, A549 cells were co-cultured with RAW264.7 and analysed for IL-10. Indeed, mouse IL-10 was detected in these supernatants (Figure 3.12). By now, it seems certain that the cytokine inducing stimulus is conserved for human and mouse cell types.

3.32 IL-12p40 but not IL-12p70 is significantly expressed in Cell/Cell co-cultures

A critical cytokine for NK and T cell activation, IL-12 has two sub units p70 and p40. The p40 sub-unit is shared with another cytokine IL-23. Co-culture supernatants of A549, ChaGoK-1, Caco-2 cells and their Conditioned Media co-cultured with adherent PBMCs were assayed for p40 sub-unit of IL-12. Tumor cells only and not their conditioned media induced significant IL12p40 from adherent PBMCs, shown in **Figure 11**. Of the tumor cells assayed, A549 induction of IL-12p40 was most potent. Like IL-10, IL-12p40 is not detectable at early time points but is expressed almost simultaneously with IL-10. This is interesting because it indicates similar mechanisms of control in cell-cell co-cultures.

3.33 IL-1β and IL-12p70 were not detectable in these co-cultures

We also assayed for IL-1β, an important pro-inflammatory cytokine and p70 sub-unit of IL-12 in co-culture supernatants but could not detect any expression at any of the time points that we have looked at. This suggests that IL-12p40 detection could well be indicative of IL-23 expression, a known anti-inflammatory cytokine.

3.4 Toll-like receptor (TLR)-2 engagement on monocytes triggers cytokine responses

3.41 Tumor necrosis factor (TNF)-α expression is completely abrogated by blocking TLR-2 on Monocytes

TNF-α and other major cytokine secretion from cells of immune system is attributed to the activation of MyD88 signalling pathway. As such, the release of all the cytokines described thus far could involve TLR mediation. To unravel the receptor on monocytes, with the presumption of TLR involvement, to which the stimulus/ligand for TNF-α induction binds, THP-1 cells were blocked with 10μg/ml of anti-TLR-2 antibody (eBiosciences) for 30 minutes before co-culture. TLR-2 blocking on THP-1 cells

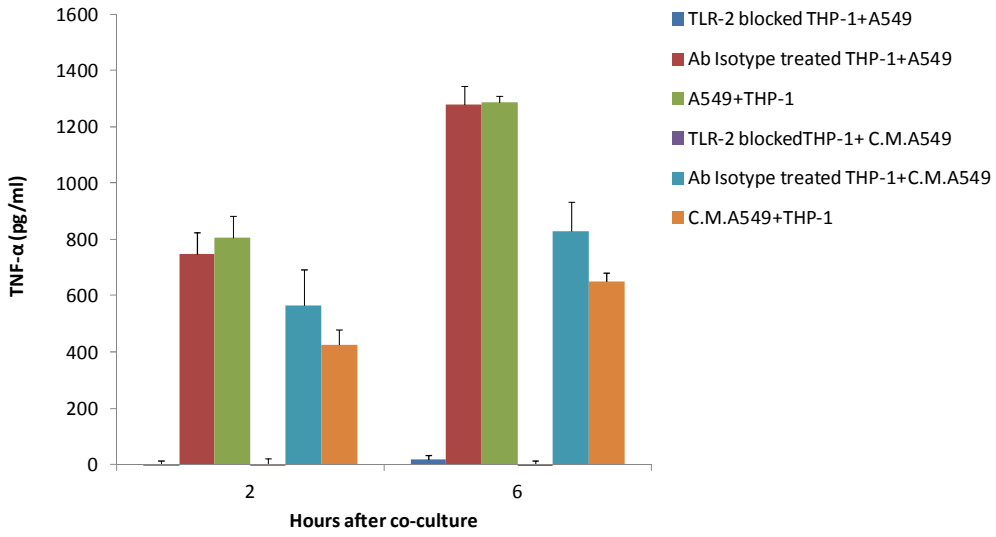

Figure 3.14: TNF-α in co-cultures of TLR-2 blocked THP-1 (5 x 10^4) +A549 (10^4) cells/conditioned medium (C. M.) of A549 cells. *Data were represented as Mean Concentration of TNF-α ± SD. Values were considered as statistically significant for $p < 0.05$ from atleast five independent experiments.*

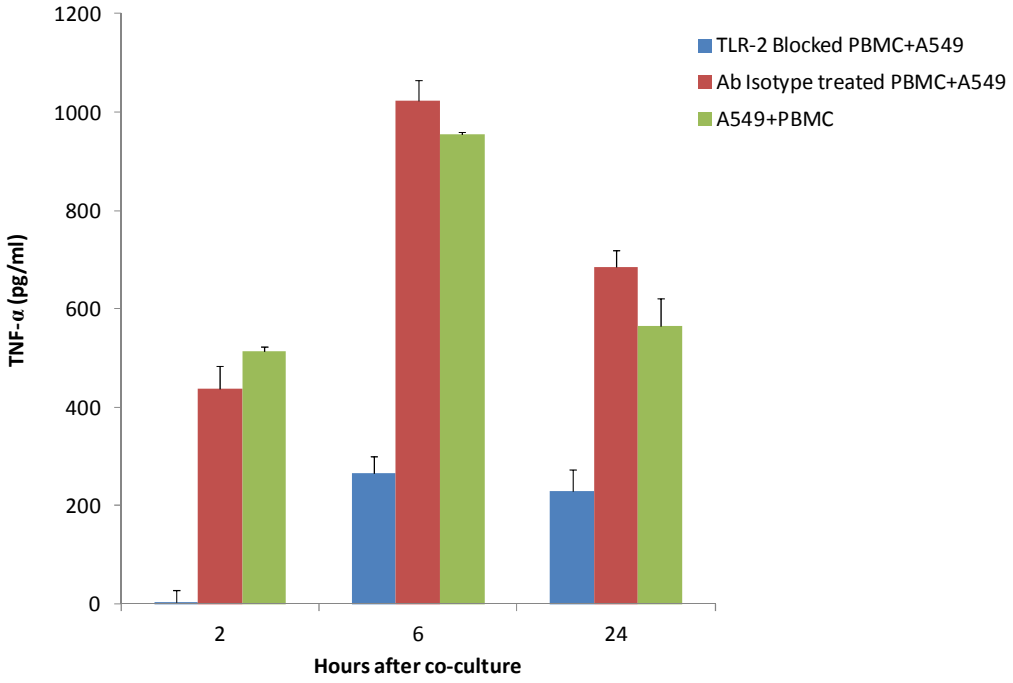

Figure 3.15: TNF-α in co-cultures of TLR-2 blocked adh. PBMCs ($2.7*10^5$) +A549 ($5*10^4$) cells. *Data were represented as Mean Concentration of TNF-α ± SD. Values were considered as statistically significant for $p < 0.05$ from atleast five independent experiments.*

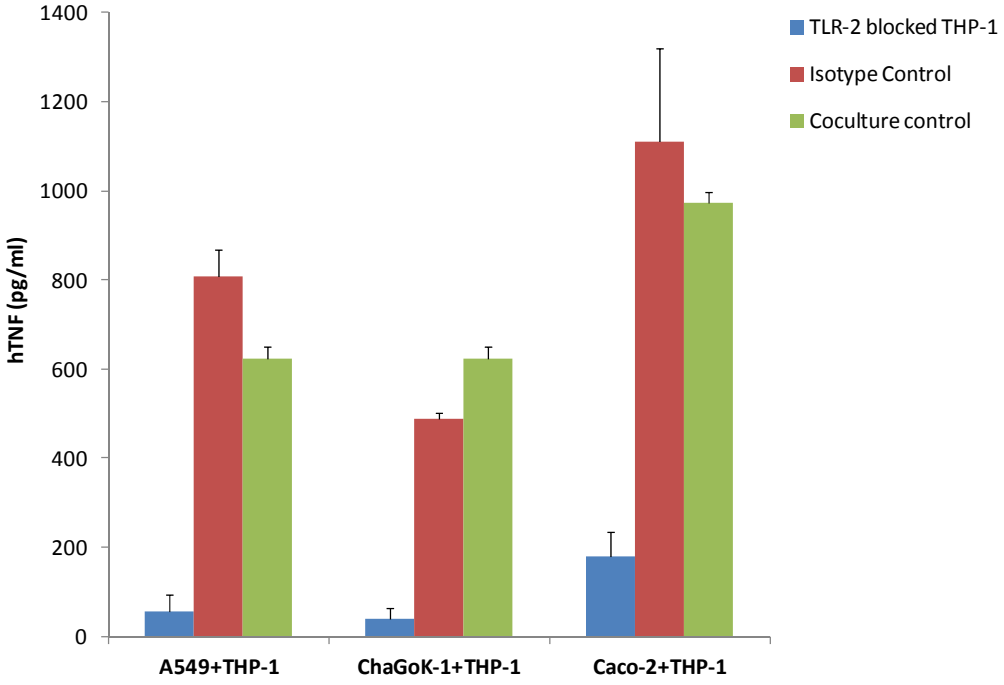

Figure 3.16: TNF-α in co-cultures of TLR-2 blocked THP-1 (2 x 10^4) + A549/ChaGoK-1/Caco-2 (12x 10^4) cells. *Data were represented as Mean Concentration of TNF-α ± SD. Values were considered as statistically significant for $p < 0.05$ from atleast five independent experiments.*

completely abrogated TNF responses triggered by A549 cells as well as Conditioned Medium of A549 cells (Figure 3.14). Monocytes were blocked for TLR-4 (data not shown) but it had no effect on TNF-α expression (see section 2.3.12).

Similar to THP-1 cells, TNF-α expression induced by tumor cells was compromised from PBMCs when blocked with 2µg/ml of anti-TLR-2 antibody for 30 minutes before co-culturing with A549 cells (Figure 3.15). Thus, it is clear that the stimulus from tumor cell engages TLR-2 to activate TNF-α expression from monocytes.

The abrogation of TNF responses was also achieved in ChaGoK-1 and Caco-2 co-cultures, by blocking of THP-1 cells with 2µg/ml of anti-TLR-2 antibody (Figure 3.16) implying that all tumor cells expressed TLR-2 agonist(s) to initiate TNF-α expression.

3.42 IL-6 expression is partially obviated by blocking TLR-2 on adherent monocytes (macrophages):

To identify the binding partner of the IL-6 inducing stimulus/ligand(s) on PBMCs in the co-cultures, adherent PBMCs were first blocked with 2µg/ml of anti-TLR-2 antibody for 30 minutes before co-culturing with A549 cells. The response, shown in Figure 3.17, was obviated, though partially. It is evident that TLR-2 binding of the ligand(s) leads to IL-6 response from monocytes/macrophages. Thus, it seems that ligand(s) for IL-6 and TNF induction act through engaging of TLR-2. The partial blockade by anti-TLR-2 could be attributed to autocrine expression of IL-6 by tumor cells.

3.43 IL-10 induction is subverted by TLR-2 blocking of monocytes

Considering that TNF and IL-6 were induced by engaging TLR-2 on Monocytes (macrophages), we checked whether TLR-2 was the receptor for IL-10 triggering stimulus too. Again, PBMCs were blocked with 2µg/ml of anti-TLR-2 antibody for 30 minutes before co-culturing with A549 cells. Indeed, IL-10 expression was subverted by TLR-2 blockade (Figure 3.18), indicating that such a response is TLR-2 mediated.

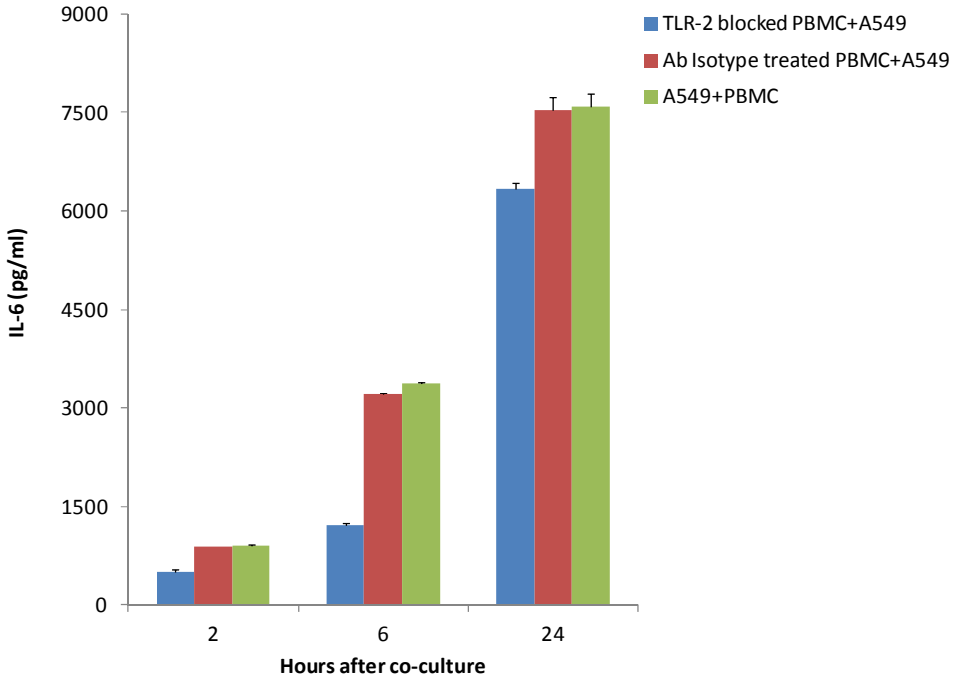

Figure 3.17: IL-6 expression in co-cultures of TLR-2 blocked adh. PBMCs ($2.7x10^5$) +A549 ($5x10^4$) cells. *Data were represented as Mean Concentration of IL-6 ± SD. Values were considered as statistically significant for $p < 0.05$ from atleast five independent experiments.*

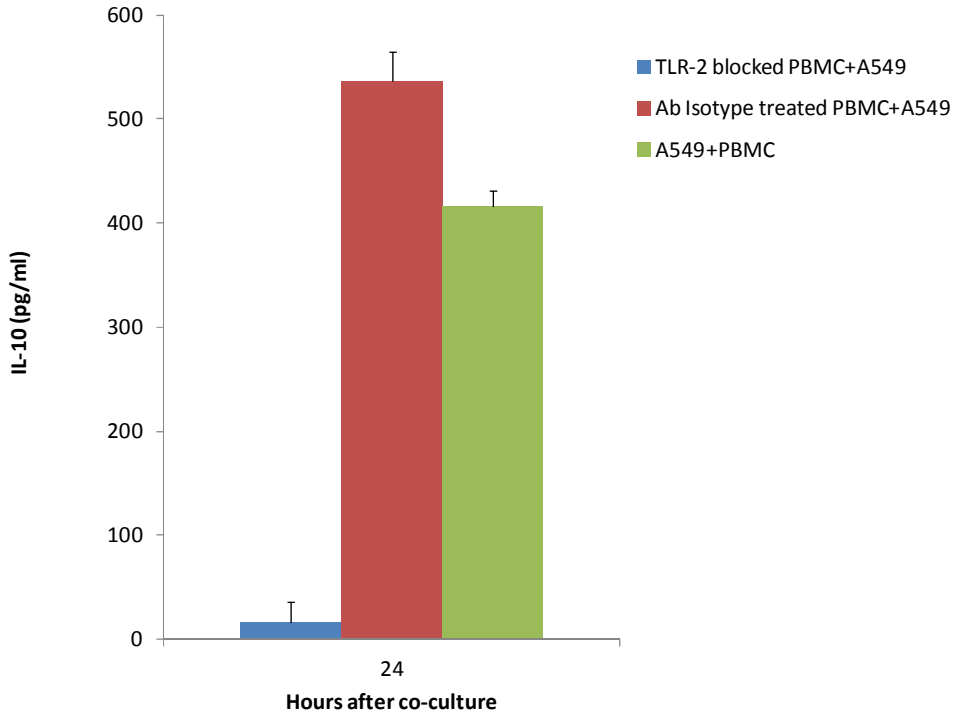

Figure 3.18: IL-10 expression in co-cultures of TLR-2 blocked adh. PBMCs ($2.7x10^5$) +A549 ($5x10^4$) cells. *Data were represented as Mean Concentration of IL-10 ± SD. Values were considered as statistically significant for $p < 0.05$ from atleast five independent experiments.*

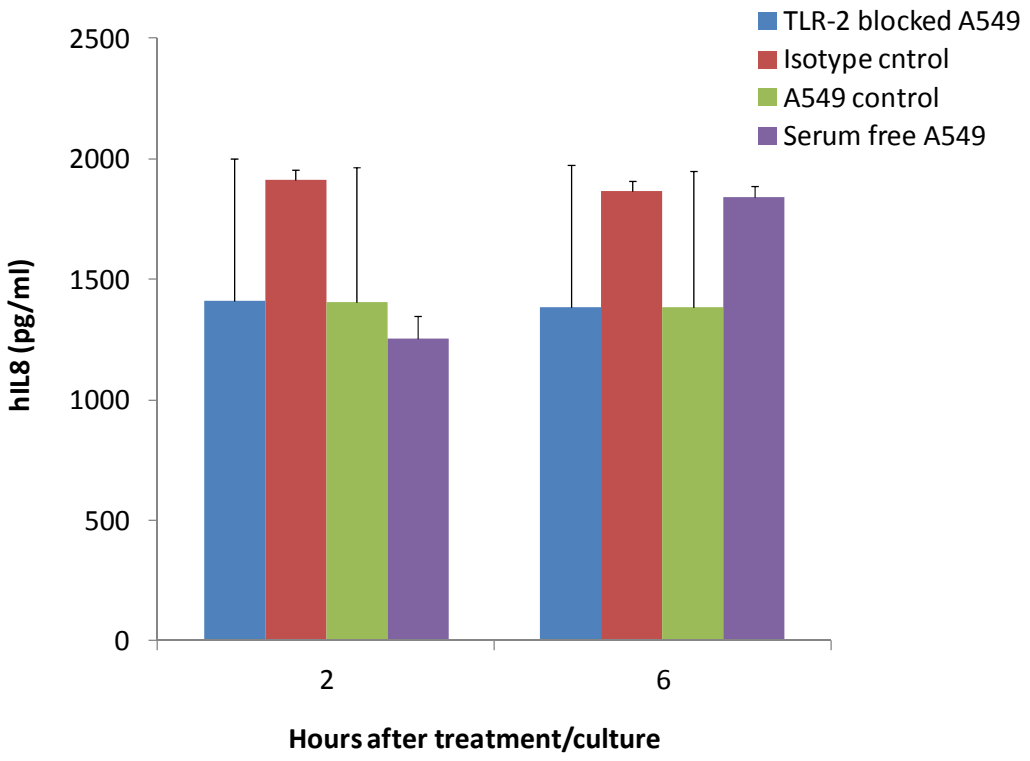

Figure 3.19: IL-8 expression in co-cultures of TLR-2 blocked A549 (10^4) cells/serum free medium of A549. *Data were represented as Mean Concentration of IL-8 ± SD. Values were considered as statistically significant for $p < 0.05$ from atleast five independent experiments.*

3.44 Constitutive IL-8 expression by A549 is not affected by TLR-2 blocking

A549 cells express IL-8, a pro-inflammatory and pro-angiogenic cytokine, constitutively (from previous experiments). IL-8 is known to attract neutrophils and NK cells. To evaluate the possibility of TLR-2 agonist based activation of A549 cells to bring about IL-8 expression and thus forming an active autocrine loop, A549 cells were blocked with 2µg/ml of anti-TLR-2 antibody for 30 minutes and then assayed for IL-8 at various time points (Figure 3.19). However, TLR-2 blocking did not affect the constitutive expression of IL-8 from A549 cells.

3.5 TLR-2 agonistic activity of tumor cells is regulated by MAP Kinase pathway but is independent of EGFR and Ras signaling

3.51 EGFR and Ras independent pathway regulates the TLR-2 agonistic activity of tumor cells

Mutations that lead to EGFR (tyrosine kinase) overexpression (known as upregulation) or overactivity have been associated with a number of cancers, including lung cancer. Mutations, amplifications or misregulations of EGFR or family members are implicated in about 30% of all epithelial cancers. On the other hand, Ras proteins function as binary molecular switches that control intracellular signaling networks. Ras-regulated signal pathways control such processes as actin cytoskeletal integrity, proliferation, differentiation, cell adhesion, apoptosis, and cell migration. Of the major intracellular pathways involved in tumorigenesis is Ras mediated pathway. Ras is the most common oncogene in human cancer - mutations that permanently activate Ras are found in 20-25% of all human tumors. Mutations or hyperactivity of EGFR and Ras tend to be mutually exclusive. As such these both were suspected to be involved in control of TLR-2 agonist(s) from tumor cells.

Pre-treating the representative tumor cells with EGFR (tyrosine kinase) inhibitor, AG1478 and Ras inhibitor, FTS , followed by co-culture with monocytes the expression of TNF-α was studied. No significant change in TNF-α expression was observed (Figure 3.20). Pharmacological inhibition of both EGFR and Ras did not affect the TNF-α inducing TLR-2 agonist(s) activity of tumor cells. This indicates involvement of some

other signaling pathway in regulation of factor(s) from tumor cells that induce cancer related inflammatory responses.

3.52 Intracellular MAP Kinase pathway regulates TLR-2 agonistic activity of carcinoma cell

Major intracullar signalling pathways include Mitogen activated protein (MAP) Kinase pathway, Phosphotidylinositide (PI)-3 kinase pathway, Nuclear factor (NF)-κB pathway. To deduce the intracellualar pathway that supervise the tumor factor(s) leading to inflammatory responses , tumor cells were treated with various inhibitors seperately before co-culturing them with monocytes (see section 2.3.10). The non-toxic concentrations were standardised for each cell type before setting up co-cultures (see section 2.3.11). Expression of TNF-α was taken as indicator of induction of cancer related inflammtory reponse.

Inhibition of MEK-1 (MAP kinase) , by MEK-1 specific inhibitor PD98059, in lung origin tumor cells (A549 and ChaGoK-1) showed abrogation of TNF-α from co-culture supernatants (Figure 3.21). Pharmacological inhibition by SB202190, p38 inhibitor, also lead to partial subversion of inflammatory responses.

Interestingly, when HeLa and Caco-2 cells were pretreated with inhibitors followed by co-culture with monocytes, maximal inhibition was observed in co-cultures with PD98059 treated tumor cells (Figure 3.22). This substantiates that MEK-1 and in turn MAP Kinase is involved in relation of TLR-2 agonistic activity of carcinoma cells. Partial involvement of p38 signaling also corroborates MAP Kinase involvement in control of inflammation inducing tumor factor(s).

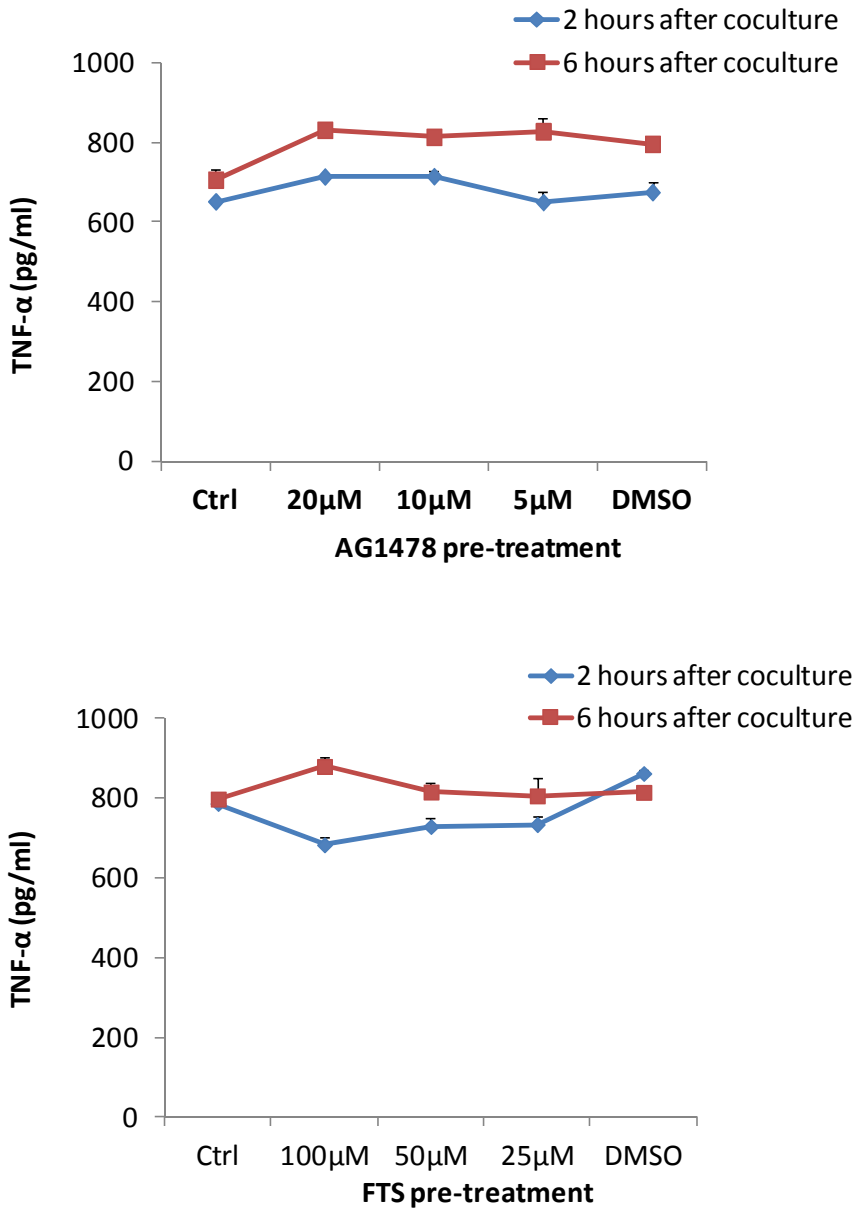

Figure 3.20: Effect of EGFR and Ras inhibition on TLR-2 ligand regulation in tumor cells. (AG1478 (tyrphostin) – EGFR inhibitor; FTS – Ras inhibitor). *Data were represented as Mean Concentration of TNF-α ± SD. Values were considered as statistically significant for $p < 0.05$ from atleast five independent experiments.*

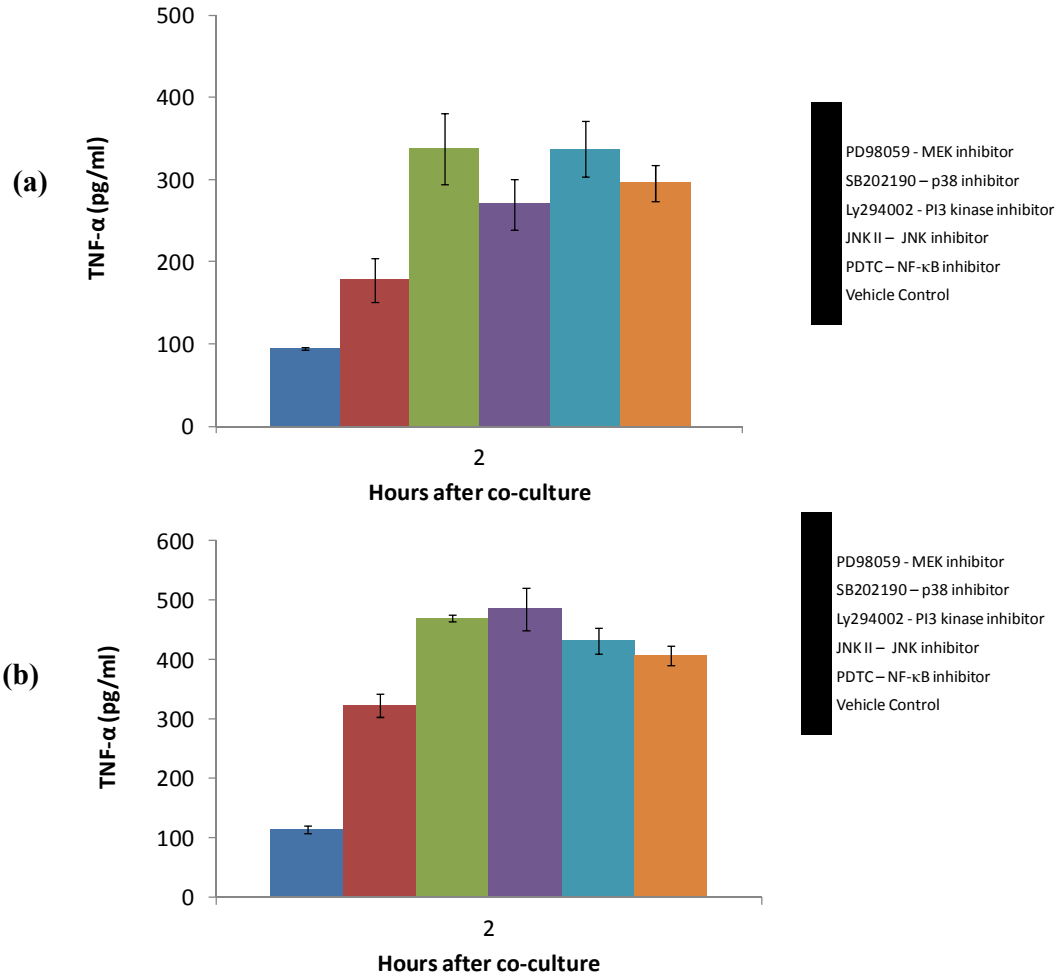

Figure 3.21: Effect of various intracellular signaling inhibitors on TLR-2 agonistic activity of (a) A549 and (b) ChaGoK-1 cells. *Data were represented as Mean Concentration of TNF-α ± SD. Values were considered as statistically significant for $p < 0.05$ from atleast five independent experiments.*

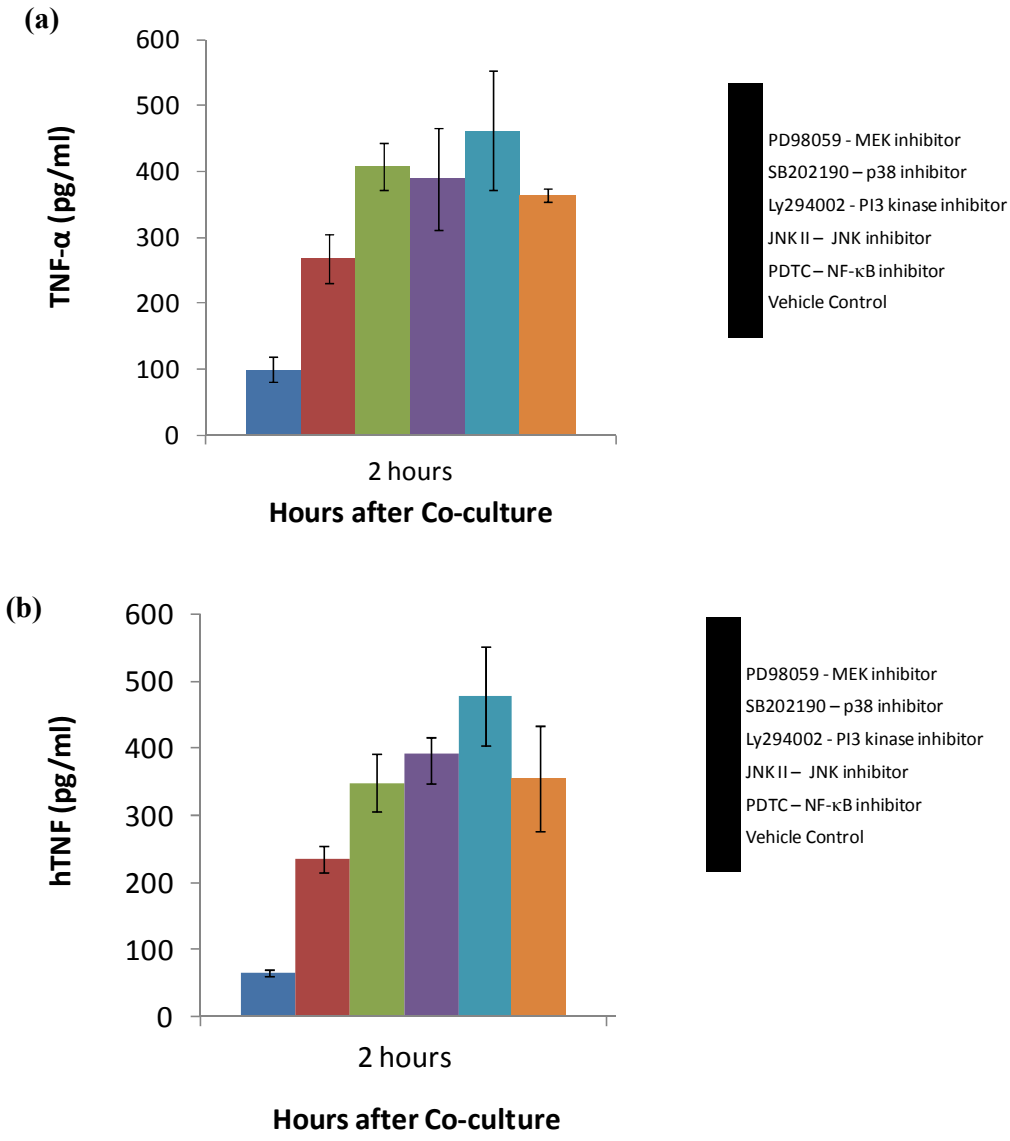

Figure 3.22: Effect of various intracellular signaling inhibitors on TLR-2 agonistic activity of (a) HeLa and (b) Caco-2 cells. *Data were represented as Mean Concentration of hTNF ± SD. Values were considered as statistically significant for $p < 0.05$ from atleast five independent experiments.*

3.6 Synergy of tumor cells and monocytes enhances proliferation of tumor cells and viability of monocytes

3.61 Co-culture of Lung carcinoma cells with monocytes enhances monocyte viability

Such keenly regulated inflammatory microenvironment is bound to affect the properties of all cells involved. After all the consideration is that tumor cells are manipulating other cells especially precursor myeloid lineage to evade immune responses. To analyze the effect of this typical inflammatory environment and continuous crosstalk with tumor cells, on monocytes, A549 cells were co-cultured with THP-1 cells for 96 hours. Under normal circumstances such prolonged cultures began to show substantial dead or dying cells.

The suspended THP-1 cells in the co-culture were separated and stained for Propidium Iodide against relevant controls (see section 2.3.9). The normal culture of THP-1 cells grown for 96 hours showed 28.2 % dead or dying cells as depicted in Figure 3.23 (b). Strikingly, the THP-1 cells from 96 hour co-cultures showed only 2.7 % dead or dying cells as depicted in Figure 3.23(a). Fresh THP-1 culture of 24 hours showed only 0.9 % unhealthy cells represented in figure 3.23(c). This indicates that continuous interaction of monocytes with tumor cells leads to better viability of monocytes.

3.62 TNF rich co-culture supernatant induces proliferation, invasiveness of tumor cells

The wound-healing assay is simple, inexpensive, and one of the earliest developed methods to study directional cell migration *in vitro*. This method mimics cell migration during wound healing *in vivo*. The basic steps involve creating a "wound" in a cell monolayer, capturing the images at the beginning and at regular intervals during cell migration to close the wound, and comparing the images to quantify the migration rate of the cells. It is particularly suitable for studies on the effects of cell-cell interactions on cell migration.

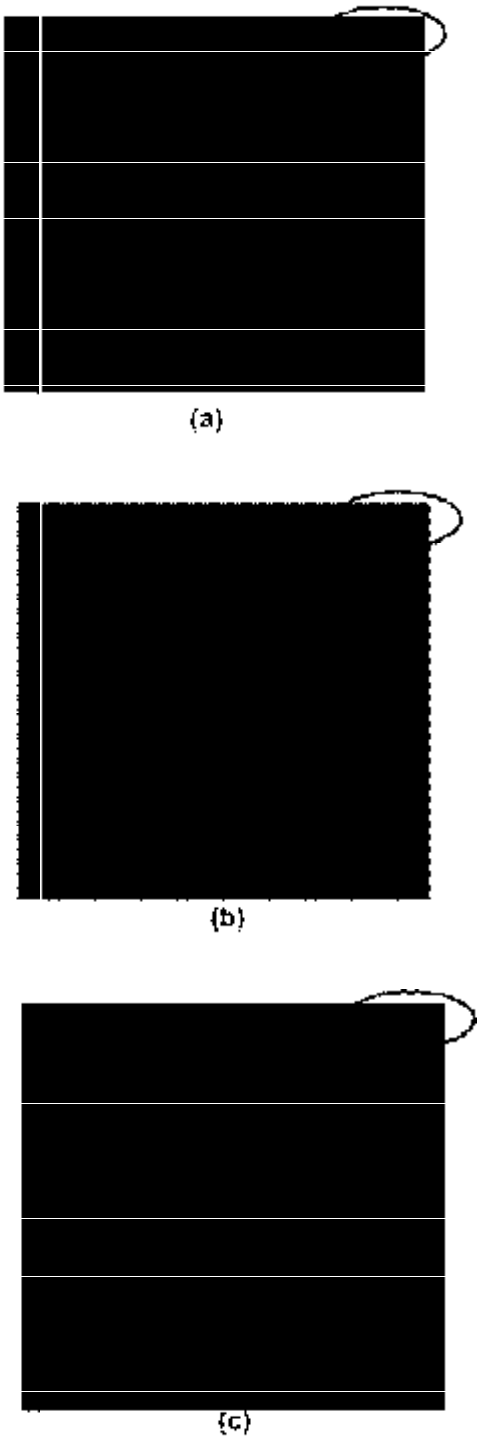

Figure 3.23: PI staining of THP-1 cells (a) from Co-cultures at 96 hours (b) from control cultures at 96 hrs (c) from fresh control at 24 hours. (Red encircled value denotes the percentage of cells that stained for Propidium Iodide).

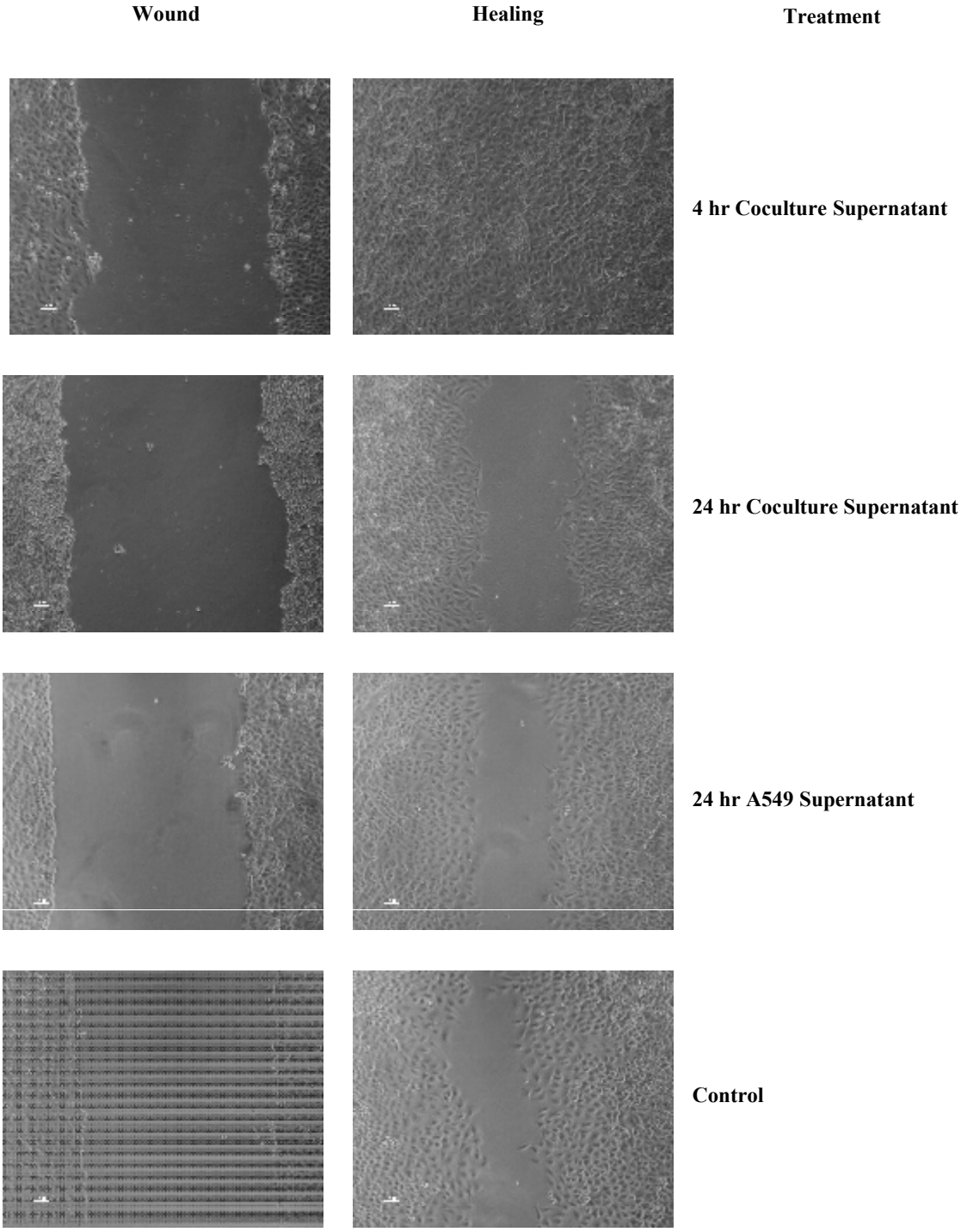

Figure 3.24: Wound healing in A549 cells when treated with co-culture and control supernatants of various time points. (Healing after 24 hours).

To study the effect of co-culture interactions on tumor cells wound healing of A549 was evaluated against appropriate controls (see section 2.3.8). The left hand panel of Figure 3.24 depicts the wound made by plastic tip of 200µl pipette tip while right hand panel depicts the healing after 24 hours after the wound.

The A549 cells treated with 4 hour A549-THP-1 co-culture supernatant shows significantly better healing than the rest (Figure 3.24). Cells treated with 24 hour co-culture supernatant or 24 hour A549 only supernatant or untreated control showed marginal healing. We have already evaluated that the 4 hour co-culture supernatant is rich in TNF-α and IL-6 cytokines, implying that these cytokines might be involved in enhanced proliferation and invasiveness of the tumor cells. The 24 hour co-culture supernatant which contains cytokines like IL-10 does not seem to affect the migration properties of the tumor cells. Indeed, IL-6 activates STAT-3 pathway, a known mechanism to initiate cell proliferation.

3.7 TLR-2 dependant inflammatory responses may have implications for T-cell modulations

3.71 Culture supernatants from co-cultures of Tumor and adherent monocytes induce high Interferon-γ expression from Non-adherent monocytes (T-cell blasts)

The optimal functioning of NK and T cells is the critical feature of anti-tumor responses. The tumor cells are believed to suppress or regulate T-cell activation, averting tumor cell specific responses. The Non-adherent population from the buffy coat of Ficoll Paque treatment of blood cells mostly consists of T cell blasts or precursors (see section 2.3.7). The Non-adherent monocytes (T cell blasts) were treated with Co-culture supernatant, Conditioned Medium treated macrophage culture supernatant and Conditioned Medium from tumor cells and after activating them with 10µg/ml anti-CD3antibody and co-stimulating with 3µg/ml anti-CD28 antibody. The culture supernatants were collected at 48 hour and 78 hour time points and assayed for IL-2, IFN-γ and IL-10. Curiously, no IL- 2 was detected in the culture supernatants (not shown here). Upon activation, IFN-γ was expressed in huge amounts by non-adherent monocytes. Non adherent monocytes (T cell blasts) treated with Co-culture supernatants showed enhanced expression of IFN-γ (Figure 3.25).

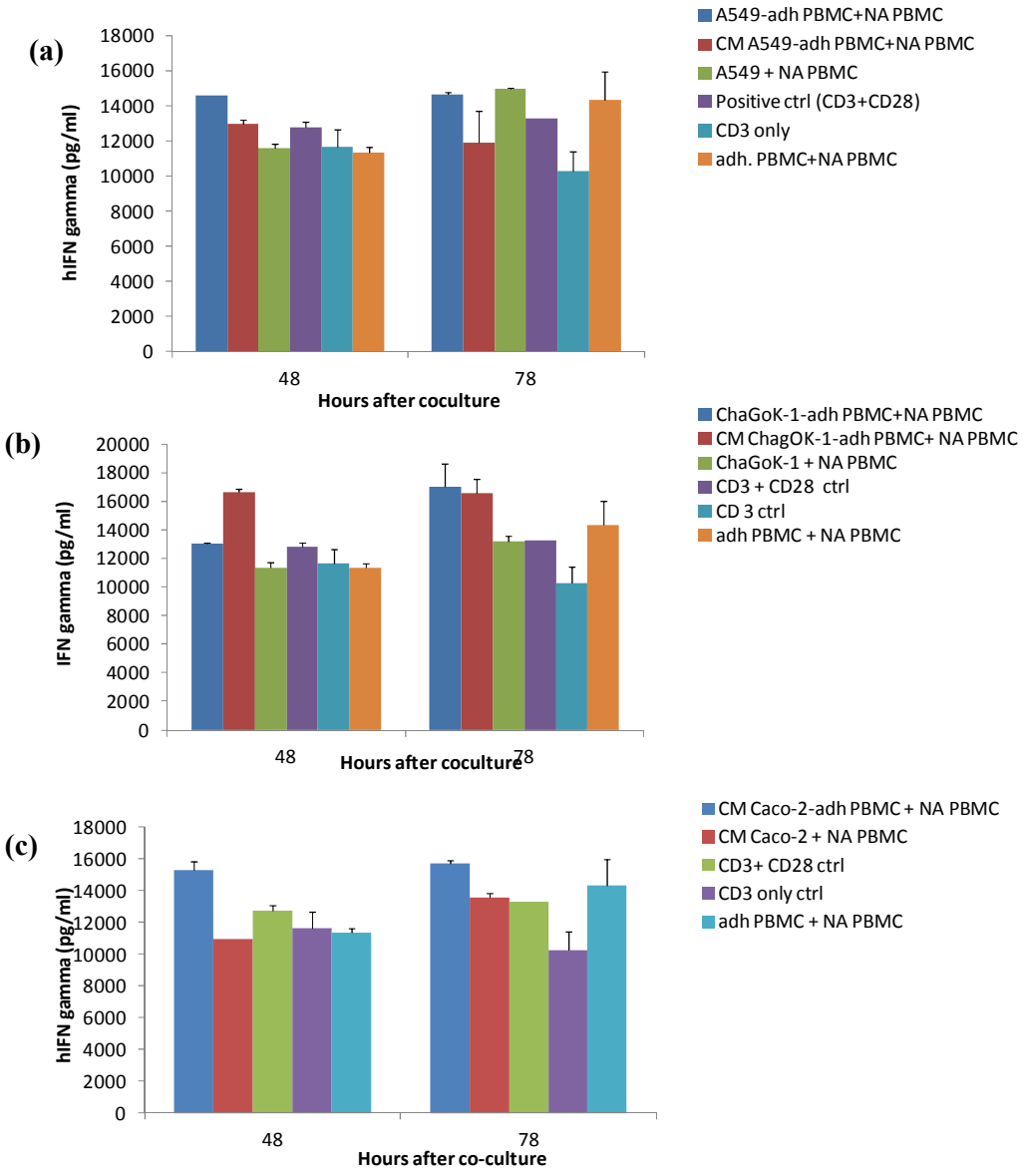

Figure 3.25: IFN-γ expression in Culture Supernatants from Non-Adherent PBMCs co-cultured with Conditioned Medium, CM (30 hour) of (a) A549, (b) ChaGoK-1, (c) Caco-2, + adherent PBMC co-culture; 10µg/ml anti- CD3 and 3µg/ml anti-CD28 antibody coated. *Data were represented as Mean Concentration of IFN-γ ± SD. Values were considered as statistically significant for $p < 0.05$ from atleast five independent experiments.*

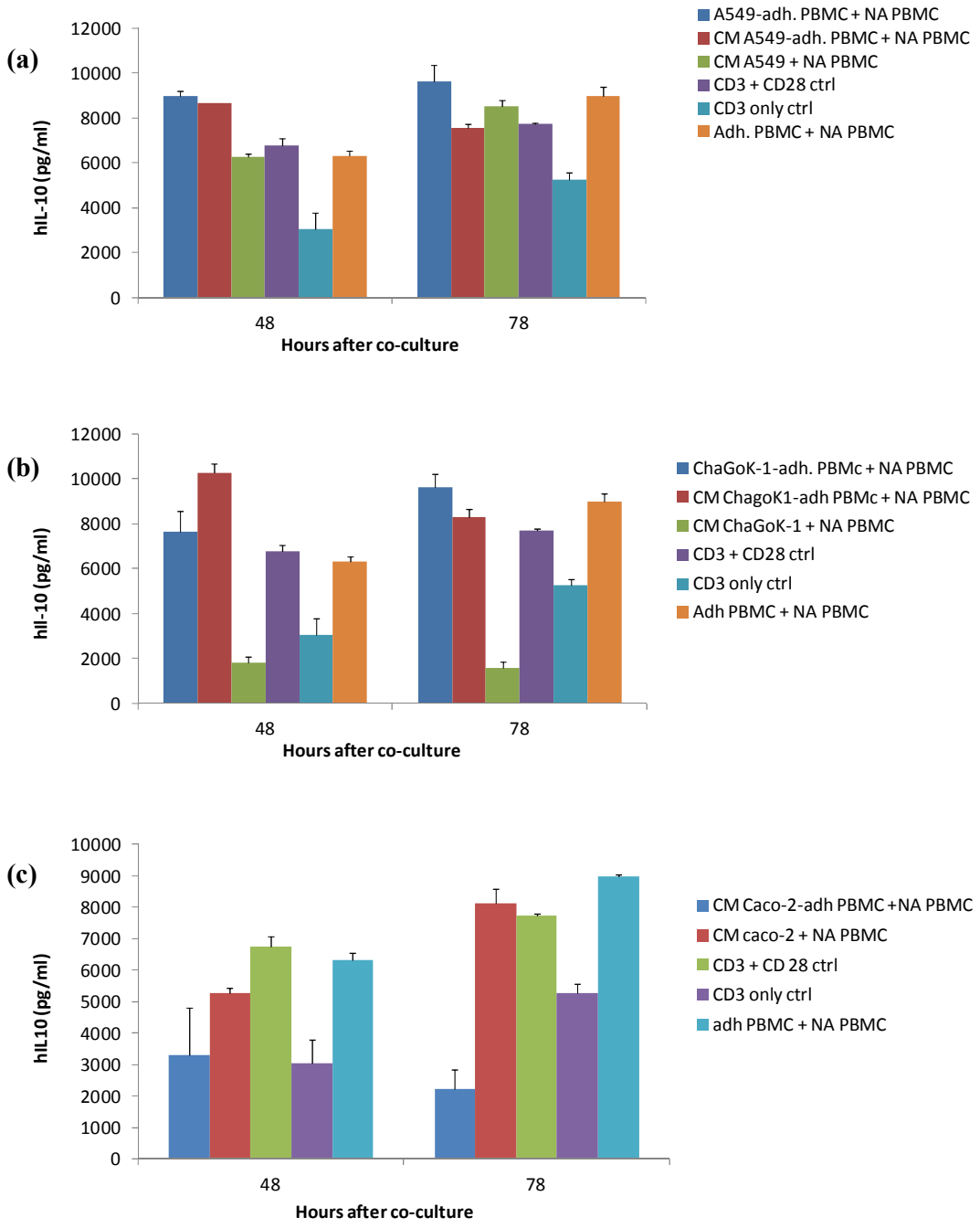

Figure 3.26: IL-10 expression in Culture Supernatants from Non-Adherent PBMCs co-cultured with Conditioned Medium, CM (30 hour) of (a) A549, (b) ChaGoK-1, (c) Caco-2, + adherent PBMC co-culture; 10µg/ml anti-CD3 and 3µg/ml anti-CD28 antibody coated. *Data were represented as Mean Concentration of IL-10 ± SD. Values were considered as statistically significant for $p < 0.05$ from atleast five independent experiments.*

This was observed with all Tumor cell co-cultures and even with supernatants from tumor-conditioned media treated monocytes. Non-adherent monocytes treated with Conditioned medium from Tumor cells only, showed marginal inhibition of IFN-γ responses.

3.72 Interleukin-10 is readily expressed by Non-adherent monocytes (T-cell blasts) when treated with culture supernatants from Co-cultures

IL-10, responsible for mediating Th2 responses, was also assayed in the culture supernatants from anti-CD3-CD28 antibody stimulated Non adherent monocytes treated with Tumor cell-adherent monocyte co-culture supernatant, Conditioned Medium treated macrophage culture supernatant and Conditioned Medium from tumor cells. It is observed that medium from adherent monocytes, treated or untreated, elicits potent IL-10 induction from T-cell blasts, shown in figure 3.26.

Curiously, while assaying the anti-CD3-CD28 pre-activated and treated Non adherent monocytes, it is observed that Conditioned medium of ChaGoK-1 cells directly suppressed IL-10 expression from *ex-vivo* T-cells **(**Figure 3.25). This observation points to differences between A549 and ChaGoK-1 cells, tumors of same origin, in triggering various cytokines and hence, varied immune responses from host immune cells. Admitted, these observations need further validations.

Although not definitive, but this suggests the possibility of modulation of T-cells at early stages by tumor-macrophage based inflammation which could have implications for T-cell maturation as well as effector T-cell responses.

A complex network of inflammatory mediators is involved in inflammation-associated cancers. The links between inflammation and cancer have been confirmed in a number of experimental models, e.g., in liver and colon cancers. Macrophages are key cells in chronic inflammation and are recruited from monocvtes. Tumor-Infiltrating Macrophages (TIMs) are known to constitute a large part of tumors especially carcinomas (tumors of epithelial origin) and it is established that these TIMs are recruited from the circulating monocyte pool. The macrophages once in the tumor vicinity are 're-educated' for a phenotype that is beneficial for tumor growth/progression/metastasis, which forms a part of a broader concept of Immunoediting. In neoplasia, monocytes are recruited into the tumor from the peripheral circulation and are usually polarized toward an M2- like phenotype. However, there is little information on how macrophages attain this M2-like phenotype. Initial understanding is that the tumor favoring phenotype is brought about by the crosstalk through the microenvironment of the tumor cells and is mainly determined by the cytokine/chemokine milieu i.e. combination of pro-inflammatory and anti-inflammatory cytokines. The regulation of these cytokines skews the macrophage phenotype from Classical 'M1' to somewhat suppressed 'M2', also called TAM (Tumor-Associated Macrophage) phenotype. Cytokines and chemokines influence movement of malignant cells and supporting stromal cells in primary tumors, and spread of cancer cells. This fine-tuned network influences the composition and phenotype of infiltrating immune cells and contributes to immunosuppressive polarized Th2 response. Some cytokines like TNF, IL-6, IL-10, IL-12, IL-1, IL-8, TGF-β are predominantly seen to be involved in generating such a milieu. Another important consequence of such cytokine milieu is suppression/regulation anti-tumor responses from Cytotoxic (NK & T) cells. This suppression is evaluated by studying the expression of cytokines specific to these cells like IL-2, IFN-γ, IL-10.

In this study, we demonstrate that the interaction of carcinoma cells of different origins and monocytes, an important immune cell, is a two-way process and that cancer cells are also capable of modulating the monocyte phenotype *in vitro*. Following co-culture there are dynamic changes in monocyte expression of mediators such as TNF-α, IL-6, IL-10, IL-12p40, generating a cytokine milieu in the typical of alternative activation. We have evaluated the cytokine expression when immune cells are present in

the immediate vicinity of the carcinoma cells especially lung carcinoma cells of human origin.

Tumor necrosis factor-α is produced by tumor cells or inflammatory cells promoting tumor survival. Indeed, TNF-α has been demonstrated to promote tumorigenesis as TNF-α-deficient mice or mice treated with anti-TNF-α antibodies are largely protected from the chemical induction of skin papillomas (Moore *et al*., 1999; Scott *et al*., 2003). TNF-α may also directly contribute to neoplastic transformation (Szlosarek *et al*., 2006). In humans, higher concentrations of TNF-α are found in the serum of cancer patients compared to control subjects, and correlate with decreased prognosis (Szlosarek and Balkwill, 2003). The fact that TNF-α is induced very early in the co-culture of tumor and monocytes emphasises the necessity of TNF-α in modulation of immune response against tumor cells. However, the amount of TNF-α required to bring out any tumor promoting modulations during tumor cell development would not remain the same and the same is underlined by strict regulation of TNF-α expression in all the experimental models shown in this work. The ability of TNF-α to further initiate further response might be dependent on achieving some kind of optimal or threshold levels as indicated by the kinetics of TNF-α triggered in all tumor types under study in this work.

The involvement of both IL-6 and STAT3 in malignant cell survival and proliferation has been well documented in numerous experimental systems (Aggarwal *et al*., 2006; Rose-John *et al*., 2006). Through the activation of genes involved in cell cycle progression and suppression of apoptosis, IL-6 can directly protect tumor cells from apoptosis. This work is indicative of acquiring such features for tumor cells as well as for monocytes. The unusually high concentration of IL-6 and its persistence in the co-culture supernatants could be enabling the properties of proliferation and apoptosis suppression. IL-6 has also been shown to act as an autocrine growth factor for tumors (Baffet *et al*., 1991). Indeed, our assays confirm autocrine secretion of IL-6 but seem to be triggered by monocyte sensing, indicating intricate IL-6 based mutual crosstalk of the two cell types. It is known that IL-6 is essential in the initiation and maintenance of chronic inflammation of the colon (Atreya *et al*., 2000). Thus, profound secretion of IL-6 is definitely contributing to the inflammatory help required by various epithelial tumors. A high TNF-α and IL-6 microenvironment points to the possible dependence of tumor initiation on inflammation while also serving as a prelude to more obvious anti-inflammatory

response. The pro-inflammatory cytokine like TNF-α, IL-6 rich cultures enhanced the cell migration and proliferative properties of the tumor cells. This is in accordance with the known tumor promoting abilities of IL-6 and TNF-α.

It is suggested that IL-10 possesses immunostimulatory activity that enhances antitumor immunity (Mocellin *et al*., 2004). Although IL-10 usually exerts antitumor activity, its biological effects are not all that simple, and consistent with its ability to activate STAT3, it might also promote tumor development. Direct effects of IL-10 on tumor cells that might favor tumor growth have been reported. For example, an IL-10 autocrine and/or paracrine loop might have an important role in tumor cell proliferation and survival. IL-10 has also been shown to modulate apoptosis and suppress angiogenesis during tumor regression (Sato *et al*., 2011). These dramatically opposing effects of IL-10 might depend on interactions with either cytokines or factors found in the tumor microenvironment, as it is unlikely that IL-10 functions in isolation. In our experience, IL-10 is expressed by the monocytes when continuously cultured together with the tumor cells. It is safe to state that the late onset of IL-10 indicates that initial modulation by pro-inflammatory cytokines is necessary for eventual take over by IL-10 to exert its effects. A better understanding of IL-10 signalling is needed before its effects on tumor growth and antitumor immunity can be fully explained. IL-12p40 is a subunit of IL-12 and IL-23 cytokines but both are known to exert opposing inflammatory roles, where IL-12 is a pro-inflammatory cytokine necessary for activation of anti-tumor NK cells and IL-23 is an anti-inflammatory cytokine involved in tissue rebuilding. The peculiar facet in the milieu of presence of IL-12p40 and absence of IL-12p70, the other IL-12 sub-unit strongly suggest IL-23 presence in the tumor-monocyte microenvironment. This is well in tune with the requirements of tumor as repair properties of IL-23 would aid in tumor progression. The absence of pro-inflammatory IL-1β from the cultures additionally strengthens the argument of active modulation of inflammatory responses that benefit tumor cells. The synchronised expression of IL-10 and IL-23, both known tumor promoting and anti-inflammatory cytokines, suggests this particular inflammatory milieu as an enabling characteristic for tumor cells of epithelial origin. Macrophages respond to microenvironmental signals and represent a spectrum of M1 to M2 phenotypes. The cytokine expression provides evidence of M2 like phenotype in monocytes. This M2-like phenotype, with an IL-10^{high}IL-12^{low} expression, of recruited monocytic precursors

(Figure 4.1) is comparable to tumor associated macrophages. The tumor environment is thus, thought to educate tumor-associated monocytes toward a tumor-promoting phenotype but the mechanisms of this are not fully understood.

It naturally follows that such novel expression of cytokines must be initiated by some trigger from the tumor cells which binds to a particular receptor on the monocytes. During tumor development, inflammation may be triggered by receptors recognizing non-self molecules on tumor cells. The family of TLRs is an important mediator of the innate response, and activation of these receptors triggers the production of several molecules involved in anti-tumoral responses. Studies have indicated that TLRs have a crucial role in the development of tumours as they arise in their natural microenvironment, thus indicating an unknown aspect of tumorigenesis (Maeda *et al*, 2005; Naugler *et al*, 2007). A formal role of TLRs in initiation with concatenate inflammation is yet to be determined. A strong possibility of the receptor for the monocyte based responses to be upstream of NF-κB, led to the evaluation of Toll like receptors as candidates. Of the known TLRs in mammalian system, TLR-2 and TLR-4 have known endogenous ligands. Of course, the stimulus from tumor cells targeted TLR-2 on the monocytes as evident from blocking experiments. Nevertheless, the TLR-2 dependence for initiation of both types of cytokines underlines similarity of stimulus and mode of action. Interestingly, the engagement of TLR-2 is prevalent in all cell types and co-cultures under study, indicating universality of this phenomenon. Hence, the cytokine inducing tumor factor(s) is a TLR-2 agonist, something not known in mammalian systems.

The regulation of the expression of TLR-2 agonist(s) would constitute another characteristic of tumor cells. This regulation expectantly could be based among the known oncogenic/tumorigenic pathways in the cancer cells. Screening for pathway inhibitors elucidated the involvement of MAP kinases, especially the MEK arm of the pathway in regulating expression of TLR-2 agonist(s). MAPK pathways are comprised of a three-tier kinase module in which a MAPK is activated upon phosphorylation by a mitogen-activated protein kinase kinase (MAPKK), which in turn is activated when phosphorylated by a MAPKKK. Mitogen-activated protein kinase (MAPK) pathways are evolutionarily conserved kinase modules that link extracellular signals to the machinery that controls fundamental cellular processes such as growth, proliferation, differentiation, migration and apoptosis. As such, this pathway is strongly involved in various hallmarks

of tumorigenesis. MAP kinase pathways actually being involved in controlling of TLR-2 agonist(s) points to definitive role in tumor promoting inflammation. What is striking is the exclusion of Ras and Tyrosine kinase (EGFR) activity in regulating the TLR-2 agonist activity. This opens up the possibility of lesser known MAP kinase activating moieties that may lie upstream of MEK-1 activation. Noticeably, the p38 arm is also partially involved with TLR-2 agonist(s) control. This also potentiates MAP kinase checkpoints afresh for therapeutic targeting of tumors from the perspective of tumor related inflammation. The crux of the work presented here is schematically represented in Figure 4.1. The data published in this study raises the possibility that recognition of tumor cells by macrophages, and vice versa, is important in initiating and, possibly, maintaining the cancer cytokine microenvironment, and may explain why there are abundant macrophages in the tumor microenvironment and modulated early while being recruited from monocytic pool.

In summary, the data presented here suggests that communication between tumor associating monocyte and tumor cells is based on cytokines represented in typical Cancer related Inflammation of the microenvironment. We demonstrate that cultured cancer cells of different origins promote monocyte differentiation toward a phenotype that resembles the alternatively activated state of Tumor Associated Macrophage (TAM). This switch involved a dynamic “chemical conversation” between the tumor cells and monocytes and is somewhat dependent on cell-cell contact. The novelty of this work is that we used a simple model system that shows that tumor cells actively modulate monocytes to generate tumor associated inflammatory phenotype of microenvironment *via* TLR-2 mediated cross-talk (Figure 4.1). The regulation of such synergy is not dependant on Ras or tyrosine kinase signalling but is instead MAP Kinase driven in tumor cells. This, in turn has consequences for proliferative properties of tumor cells, longevity of monocytes and perhaps, modulation of T-cell activity. This study provides a rationale for targeting monocytes and cytokines as a part of the tumor-promoting microenvironment in various carcinomas.

Even though this study provides fresh insights into tumor-monocyte interaction and the resulting inflammatory microenvironment, many unanswered and worthy questions have remained. A profound evaluation of the functional significance of each

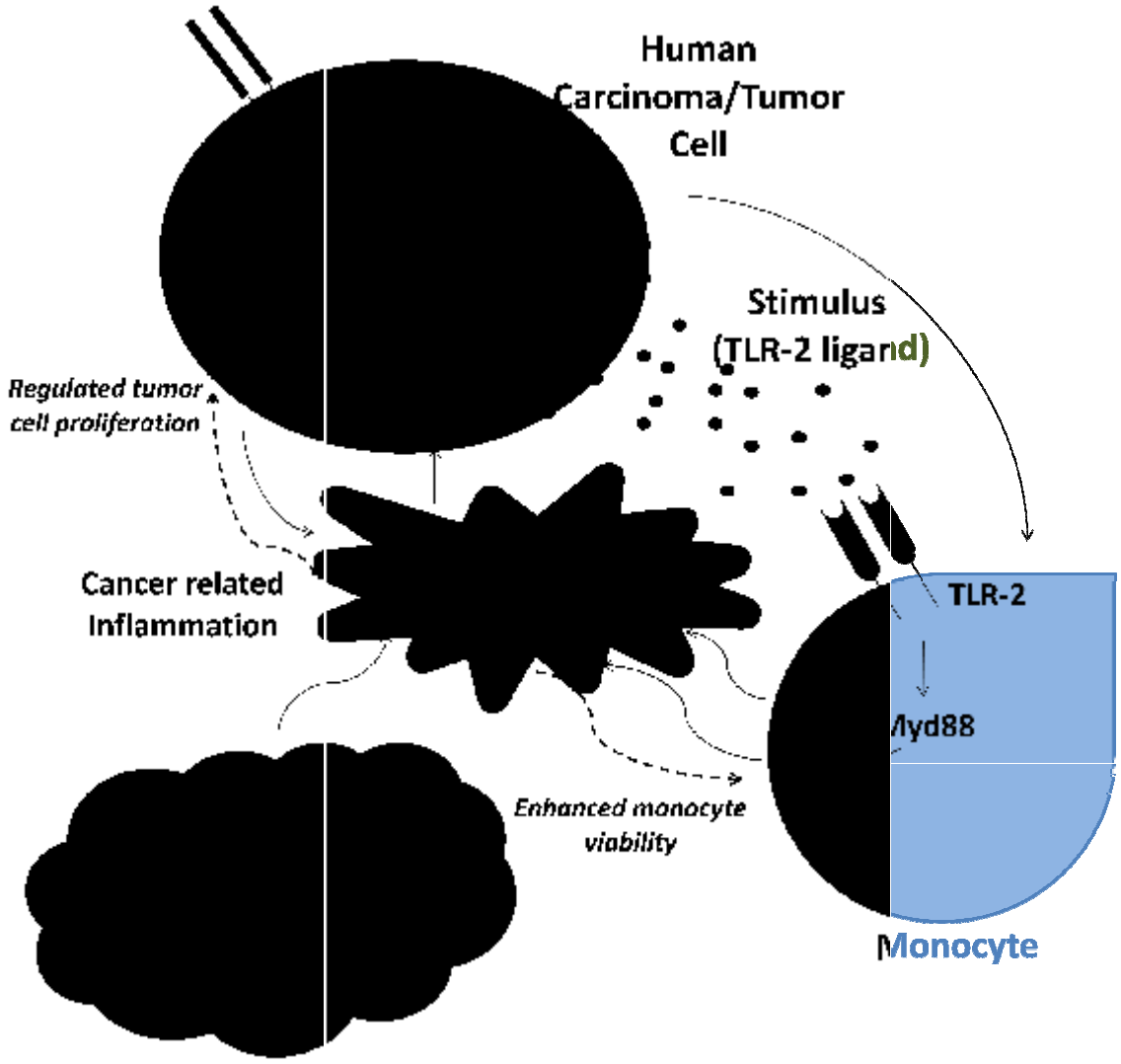

Figure 4.1: Schematic model representing TLR-2 engagement on monocytes by MAP-kinase dependant factor(s) from human carcinoma cells triggering Cancer related Inflammation.

cytokine from tumor cells and immune cells is particularly interesting. Studies to understand the similarities and differences between the various tumor cell types presented in this study would help in deducing tumor progression mechanisms in each tumor subtype. Also, the strong possibility of other cytokines and chemokines also being triggered in co-cultures as shown in this work is worth persuading. The identification and characterisation of the TLR-2 agonist(s) could be objective of immediate study. The regulatory mechanisms involved in the expression of such TLR-2 agonist(s) could provide some therapeutic check points to tackle cancer.

It remains to be seen if anti-inflammatory molecules can deliver therapeutic benefit to patients suffering from large tumors, rather than serving merely a prophylactic role potentially preventing cancer occurrence.

The conclusions from the present study can be summarized as follows:

- Human carcinoma cells trigger Cancer related Inflammation by expression of TNF-α, IL-6, IL-10, IL-12p40 but not IL-12p70 or IL-1β from monocytes.
- TNF-α and IL-6 are induced early while IL-10 and IL-12p40 are expressed only after continuous interaction of the two cell types.
- Stimulus of tumor cell origin for these inflammatory responses is cell associated as well secretory (released) in nature.
- The factor(s) inducing these inflammatory responses during tumor and monocytes synergy is tightly regulated, as indicated by kinetics of cytokine expression.
- Cancer related Inflammatory responses are generated *via* engagement of TLR-2 on monocytes.
- The expression of such typical cytokine milieu inducing TLR-2 agonist(s), from human carcinoma cells, is controlled by MAP-kinase pathway of intracellular signaling cascade.
- The TLR-2 agonistic activity of tumor cells is not dependent on activation of EGFR or Ras, the known oncogenic signals upstream of MAP kinases.
- Early inflammatory response from monocytes promotes tumor cell proliferation.
- Tumor-monocyte interaction enhances monocyte viability.

These results reveal a previously unrecognized pathway that might regulate activation of TLR2-dependent Cancer related Inflammatory responses during crosstalk of cancer and immune system.

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