Chapter 1

Review of Literature
1.1 The Immune system - an overview

The term “immunity” is broadly defined as resistance to diseases, specifically infectious diseases and refers to the global ability of host to resist invasion of foreign (non-self) antigens (Ag) from the environment. These foreign antigens would otherwise disturb integrity of the balanced and dynamic network of various preventive pathways existing in body (Hoebe K et al., 2004). Immune system consists of a complex network of interdependent cell types and organs with specialized functions. Biochemicals produced by them distinguish between self and foreign/non-self molecules and collectively protect the host from bacterial, parasitic, fungal and viral infections as well as the growth of tumor cells. The physiological function of the immune system via coordinated interaction of various specialized cells and accessory molecules produced by them to protect against infections and eradicate established infections is called the “immune response”.

1.1.1 Organs of the immune system (Parkin J, Cohen B, 2001)

a) Bone Marrow - Hematopoiesis involves formation of all the cells of the immune system derived from the bone marrow. Bone marrow-derived stem cells differentiate into either mature cells of the immune system or into precursors of cells that migrate out of the bone marrow to continue their maturation elsewhere in body. Cell population produced by bone marrow includes B cells, Natural Killer (NK) cells, granulocytes and immature thymocytes, in addition to red blood cells and platelets.

b) Thymus - Mature T cells are produced in the thymus by the process of thymic education. Immature thymocytes (prothymocytes) leave the bone marrow and migrate into the thymus, where T cells acquire specific set of receptors for maturation. Cells that express self-Ag specific receptors and might evoke a detrimental autoimmune response in host are eliminated (negative selection) and those non-reactive to self-Ags are only selected for further development. Mature and functional T cells are released into the bloodstream.

c) Spleen - The spleen is known an immunological filter of the blood, which is made up of important immune cells such as B cells, T cells, macrophages, Dendritic Cells (DCs), NK
cells and red blood cells (RBCs). Migratory macrophages and DCs not only capture foreign materials (Ag) from the blood passing through the spleen, but they also bring along with them Ags to spleen via the bloodstream and initiate an immune response by presenting the Ag to the appropriate B or T cells. In the spleen, activation of B-cells to produce large amounts of antibodies also takes place. In addition, old red blood cells are destroyed in the spleen.

d) **Lymph Nodes** - The lymph nodes function as an immunological filter for the lymph throughout the body. Lymph nodes are primarily composed of T cells, B cells, DCs and macrophages and drain out fluid from most of our tissues. Antigens are filtered out of lymph in the lymph nodes before returning the lymph to the circulation. In lymph nodes also, the macrophages and DCs capture Ags and present them to T and B cells, subsequently initiating an immune response.

e) In addition to these organs, clumps of lymphoid tissue are found in other parts of the body, including the linings of the digestive tract and the airway pathways. These tissues include the tonsils, adenoids, peyer’s patches and appendix.

1.1.2 **Innate vs. adaptive response**

The immune system is typically divided into two categories –

1.1.2.1 **The innate (or nonspecific) immunity** also called natural or native immunity refers to pre-existing, nonspecific defense mechanisms in body that comes into play immediately or within hours of exposure to Ag and provides immediate defense against toxins and pathogens (Janeway CA, Medzhitov R, 2002).

a) **Surface barriers: skin and mucosa** - The first line of defense in the innate system is provided by the skin barrier and micro-flora prevailing in mucosal membranes, which act as physical barrier to invasion. Fluids secreted onto the skin (sweat and sebum), hydrochloric acid secreted by stomach lining, saliva and tears possess potential to inhibit growth of microorganisms.
b) **Internal defenses: cells and chemicals** - The second line of defense includes primarily phagocytes and antimicrobial proteins, which attempt to control the spread of any invaders that manage to escape the first line of defense. The chief phagocytic cells include macrophages (mature monocytes that have left the bloodstream and entered into the tissues) in addition to neutrophils and rarely eosinophils (in defending against invaders too large for phagocytosis, such as parasitic worms). NK cells are another vital population, specialized in detecting and killing cancer cells and virus infected cells. In addition, infection, chemicals, heat, and physical trauma initiate the inflammatory response in tissue, which involves redness, heat, swelling, and pain in the injured or infected area. The inflammatory response begins with the release of chemicals such as histamine, kinins, prostaglandins, complement, and cytokines (small cell-signaling protein molecules including interleukins and interferons secreted by immune cells and can be classified as proteins, peptides or glycoproteins). Antimicrobial proteins such as Interferons (IFNs) are released by cells infected by viruses and are able to specially hinder reproduction of viruses. Complement system is another cascade consisting of protective chemicals, at least twenty proteins that circulate in the blood in an inactive state. Upon activation, complement system amplifies the inflammatory response, destroying infected cells.

1.1.2.2 **The adaptive (or specific) immunity** also called acquired immunity refers to Ag-specific immune response and is more complex and comparatively slower than the innate immunity. Adaptive immunity is marked by specific characteristics, such as antigenic specificity, antigenic diversity, immunological memory, self and nonself recognition and tailoring of the response to particular foreign invader (Godfrey SG, 2005).

- **Cells of the adaptive immune system**
  Three types of cells play the major roles in the adaptive immune system: B lymphocytes (B cells), T lymphocytes (T cells), and Ag-presenting cells (APCs). Immature T lymphocytes become immunocompetent in the thymus and are able to recognize specific non-self Ags. B cells are developed from lymphocytes that become immunocompetent in
the bone marrow. Immunocompetent but naive T cells and B cells migrate to the lymph nodes, spleen, and other secondary lymphoid organs where they await encounters with Ags, subsequently leading to activation. APCs engulf foreign particles (uptake), break them up (processing), present Ags from the foreign particles on their own cell surfaces and then present them for recognition by T cells for generation of an effective immune response. APCs include DCs, macrophages, NK cells and activated B cells.

Adaptive defenses

a) **Humoral immune response**

B Cells are the major cells involved in production of antibodies circulating in blood plasma and lymph, which comprise body’s humoral immunity. The humoral immune response is initiated by binding of B cell to specific Ag. This binding triggers B cell activation, division and multiplication by the process of clonal selection. Most of the cloned B cells become B-plasma cells and secrete antibodies, which recognize a unique Ag. This makes the foreign particle a target for destruction by other cells of the immune system. Some of the cloned B cells also become B-memory cells, already primed to produce specific antibodies that last in the body for long periods of time. These memory cells can rapidly proliferate, if the foreign particle is encountered again a second time. The first time the body is challenged with an Ag, it produces a primary immune response (peak of antibody production occurs about ten days after the initial challenge). If the body is exposed to the same Ag again at a later time, memory cells initiate a secondary immune response more rapidly (peaking within two or three days) and more intense (with much higher levels of antibodies) than the primary immune response.

b) **Cell mediated Immune response**

B cells generally react only to the Ags circulating freely in extracellular fluids, such as blood, lymph, and other body secretions and are not preventive against Ags residing inside cells or solid tissues. T cells, on the other hand, have the potency to bind to free Ags or Ags in their natural state. T cells are specialized to respond to Ags that have been processed and displayed on the surface of the body’s own cells. The basic process of
clonal selection and differentiation of T cells remains identical to that of B cells, differing in the mechanism of Ag recognition (Timothy KS et al., 2003). Whereas B cells are activated simply by specific binding of Ag to cell surface receptors, T cells must simultaneously recognize both Ag and self. The Ag receptors of most T lymphocytes only recognize peptide fragments of protein Ags that are bound to specialized peptide display molecules called Major Histocompatibility Complex (MHC) molecules on the surface of APCs. Class I MHC proteins are displayed by nearly all cells of the body and bind to fragments of protein (peptides) arising from within the cell (endogenous Ags) usually after infection by a virus or if the cell has become cancerous. On the other hand, Class II MHC proteins are displayed on the surfaces of mature B cells, some T cells and APCs and they bind to peptides that come from exogenous Ags that have been phagocytized and broken down in phagosomes (Michael JO, Michael JC, 1987).

A variety of different types of T cells exist, each with its own specialized function:

- **CD4 cells (helper T/Th cells)** help B lymphocytes to produce antibodies upon activation and phagocytes to destroy ingested microbes. Helper T (Th) cells stimulate the proliferation of other T cells and B cells that have already bound to specific Ag. Cytokines released by helper T cells help in proliferation of CD8+ T cells to Cytotoxic T Lymphocytes (CTL) for eradication of pathogen, via interaction of T-cell receptor (TCR) with Ag-MHC-I complex. Induction of an appropriate set of effector function during an immune response broadly depends on the differences in cytokine secretion pattern. Two CD4+ Th cell populations Th1 and Th2 act in a synergistic and cross-regulated manner for generation of an effective immune response (Berger A, 2000). Th1 subset is involved in cell-mediated functions, macrophage and Tc (T-cytotoxic) cell activation and production of opsonisation-promoting IgG antibodies. The cytokine array for Th1 branch consists mainly of IFN-γ, IL-12, TNF-β, GMCSF and IL-3. Th2 subset is mainly involved in humoral functions via B cells activation and differentiation. IL-4, IL-5, IL-6, IL-10 and IL-13 constitute the cytokines array for this subset (Mosmann TR, Coffman RL, 1989).
CD8 cells (cytotoxic T/Tc cells) are called cytotoxic, or cytolytic T lymphocytes because they kill (lyse) cells harboring intracellular microbes. Upon encountering Ag, Tc cell attaches to the target cell membrane and releases perforin molecules in order to lyse it. Cytotoxic T cells differ from NK cells, in context to specificity.

Suppressor T cells Special subset of T cells that functions to prevent or limit immune responses; these are called regulatory T lymphocytes. Suppressor T cells release cytokines that suppress T and B cell activity.

Effector and Memory T cells When naive lymphocytes recognize microbial Ags and also receive additional signals induced by microbes, the Ag-specific lymphocytes proliferate and differentiate into effector cells and memory cells. Effector cells are the differentiated progeny of naive cells that have the ability to produce molecules with potential to eliminate Ags, such as cytokines. Memory cells persist in the body for longer period of time and are stored for a delayed infection.

T cell activation

T cell activation is a two step process:

Ag binding - CD4+ Th cells bind to processed Ags attached to class II MHC proteins displayed by APCs. CD8+ Tc cells bind to Ags attached to class I MHC proteins, which may be displayed by just about any type of self cell.

Co-stimulation - Various chemicals (called co-stimulators such as cytokines, which include interferons and interleukins) produced by other cells of the immune system are critical in determining whether or not a T cell is activated. Some co-stimulators are required to complete the activation process; others actually abort the activation process.

Although the innate and adaptive immune systems play different roles in defense mechanisms, they generally act together in a coherent manner to fight against an infection and prevent the host. Interdependence between the two branches of immunity can justify co-ordination of the system in a sequential manner, resulting in complete elimination of the pathogen and maintenance of a healthy immune system.
1.2 Dendritic cells (DCs) – the immunological sentinels

Important components of the immune system have been identified step by step during the 20th century. Research in area of immunology has long been focused primarily on key players -antigens and lymphocytes, but the only presence of these two parties does not always lead to immunity. A third party consisting of the DCs system of Ag presenting cells (APCs) has been recognized as the initiator and key modulator of the immune response, which is also known as the pacemaker of the immune system (Banchereau J, Steinman RM, 1998). The DCs network is a specialized cellular system which functions by presenting the Ag to naive or quiescent T and B cells, and consequently plays a central role in the induction of T cell and B cell mediated immune responses. DCs comprise an essential arm of the immune system and are established as initiators of immune responses, potent stimulators of T cells and inducers of tolerance. Often termed as "professional APCs", they have a dual role in orchestrating an immune response mediated by the amplification of innate immune responses as well as activation of adaptive immune responses. As a bridging link between innate and adaptive immune branches, DCs are recognized as immunological sentinels with a central role in health and disease.

1.2.1 History of DCs

In 1868, DCs were first identified in the epidermis and named as “Langerhans cells” (LCs) by Paul Langerhans, who initially thought they were part of the nervous system (Langerhans P, 1868, Jolles S, 2002). The epidermal layer of the skin has a rich network of dendritic cells. Dendritic cells (DCs) were first described in 1973 by Ralph Steinman and Zanvil Cohn at Rockefeller University as the cell type that is almost singularly responsible for commanding the efforts of all other immune cells. They observed a subpopulation of cells in the mouse spleen with a strikingly different dendritic shape (Steinman RM, Cohn ZA, 1973-1974). While observing the heterogeneous cell population in mouse spleen comprising of mononuclear phagocytes, granulocytes and lymphocytes, a fourth variety of adherent, nucleated and large stellate cell with morphological features markedly distinct from the other cells were noticed by Steinman.
The cytoplasm of this large cell was particularly arranged in form of pseudopods of varying length, width, form and number, resulting in a variety of cell shapes ranging from bipolar elongated cells to elaborate, stellate or "dendritic" ones. These cells were nonphagocytic, loosely adherent and of low buoyant density. Over the years, immature DCs have been identified as stellate shaped cells that actively produce a variety of cell processes, including spiny dendrites, bulbous pseudopods and lamellipodiae or veils in maturing DCs.

(Steinman RM, Cohn ZA, J Exp Med, 1973)

Figure 1.1 - Phase contrast micrographs of DCs. Dendritic cells were isolated from peripheral lymphoid organs of mice and fixed in glutaraldehyde. (a, b) - spleen, (c) - cervical lymph node, (d) - peyer's patch.

The shape and motility of DCs perfectly match their unique and specialized functions, which are capturing and processing Ags and presenting them to Ag-specific T cells. In fact, no other blood cell exhibits the characteristic shape and motility that gives rise to the term 'dendritic' cell. *In situ* DCs are stellate or tree-like cells (Greek, *dendron*, tree) present in the interfaces between our bodies and the environment such as the skin, airways and lymphoid organs (Figure 1.2a). When isolated and spun onto the slides, DCs are found to display many fine dendrites (Figure 1.2b). Under an electron microscope, these processes are observed to be long (>10 μm) and thin, either spiny or sheet-like.
Phase-contrast microscopy shows DCs with extending large, delicate processes or veils radiating outwards from the cell body (Figure 1.2d).

(Figure 1.2c). Phase-contrast microscopy shows DCs with extending large, delicate processes or veils radiating outwards from the cell body (Figure 1.2d).

**Figure 1.2 - The unusual shapes of DCs.** (a) DCs in a sheet of epidermis (MHC II stain), (b) in cytospins stained for surface MHC II, (c) by scanning electron microscopy, (d) in the live state by phase-contrast microscopy.

The discovery of DCs by Steinman and Cohn marked the beginning of modern era of "dendritic cell research" by showing that these cells are a new class of white blood cells with a number of distinctive features and functions. Following their discovery 40 years ago, DCs have been extensively studied for their incredible role in immunology and many interfaces with modern medicine. This unique cell population has been deeply investigated because of the increased realization of its importance in immunoregulation. Research has shown possibilities of exploiting them for biomedical purposes such as critical adjuvants in vaccines for prevention of microbial infection, allograft rejection and treatment of cancer and autoimmune diseases (Banchereau J et al., 2000). In 2011, Ralph Steinman known as “father of DC research” was awarded the nobel prize for discovery of
dendritic cells and their defining unique capacity to activate and regulate the immune responses.

1.2.2 Classification of DCs

DCs were initially identified to be located primarily in lymphoid organs including the thymus, spleen and lymph nodes. Subsequently they were described in more tissues and now believed to be widely distributed throughout the body, including all the non-lymphoid tissues and organs. Most of the organs possess their own resident DC populations. Although the principal function of DCs appear to be overall activation of T cells, their diverse anatomic locations indicate that they have distinct and specific strategies for regulation of their specialized functions. Dendritic cells are distributed in most tissues and in particular, in tissues that interface with the external environment, where micro-organisms can enter and infect the host.

On the basis of their tissue location, DCs can be broadly classified as (Makala LH, Nagasawa H, 2002):

a) Interdigitating DCs – in the dome and inter-follicular areas of lymphoid organs.
b) Veiled cells – in afferent lymph.
c) Peripheral blood DCs – in circulation.
d) Dermal DCs – in dermis of skin.
e) Langerhans cells – in epidermis of skin.
f) Interstitial DCs – interstitial connective tissues of non-lymphoid organs.
g) Follicular DCs – in B-cell areas (follicles) of the spleen, lymph nodes and peyer's patches.

1.2.3 Origins and development of DCs

DCs are widely distributed throughout the body, although they comprise only a small proportion (0.1 - 1%) of the cells in different lymphoid and non-lymphoid tissues. Within the past decades, there have been important advances in understanding the DC biology.
This has been possible by the ability to culture and propagate large numbers of DCs using defined set of growth factors. DCs are not a single cell type, but a heterogeneous collection of cells that have arisen from distinct, bone marrow-derived hematopoietic lineages (Caux C et al., 1996, Cella M et al., 1997). To date, at least three different pathways have been described. The emerging concepts are that each pathway develops from unique progenitors. Particular cytokine combinations guide the developmental events within each pathway and cells developing within a particular pathway exhibit distinct specialized and unique functions (Reid CD, 1997). The ability to propagate subtypes of DC from early progenitors has been critical in assessing their developmental and functional characteristics. Using immunohistochemistry and flow cytometry techniques, different DC subsets in humans and mice have been identified and well characterized.

1.2.3.1 Murine DCs

Murine DCs have been classified into three main lineages based on various surface markers and presence in different tissues (Table 1.1):

a) **Myeloid DCs** (M-DC) were originally described by Steinman and Cohn (Steinman RM et al., 1973-1975) and characterized by their monocyic morphology. These cells arise from the same progenitors that also give rise to monocytes and macrophages and express monocyte-specific cell-surface markers. They express myeloid markers CD13 and CD33 and prime T cells to preferentially activate Th1 responses mediated by secretion of IL-12.

b) **Lymphoid DCs** were described by Suss and Shortman (Suss G, Shortman K, 1996) and this subpopulation of cells secrete the lymphokines; interleukins 4, 5, 10, and 13 (IL-4, IL-5, IL-10, IL-13). They are associated with negative selection in the thymus and mediate regulatory rather than stimulatory immune effector functions.

c) **Plasmacytoid DCs** have morphology resembling plasma cells and produce high levels of IFN-γ (Asselin-Paturel C et al., 2001).
### Phenotype of DC subsets

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#### Table 1.1 - Phenotype of different DCs subsets in mouse

**1.2.3.2 Human DCs**

In humans, DCs are found mainly as precursor or immature populations in the bone marrow and blood. More mature forms are found in lymphoid and non-lymphoid tissues (Lipscomb MF, Masten BJ, 2002). Based on the studies of skin DCs, distinct subtypes have been delineated. This includes DCs generated in vitro from CD34+ myeloid/lymphoid hematopoietic progenitors and blood DC precursors. CD34+ myeloid precursors give rise to interstitial DCs and langerhans DCs. On the other hand CD34+ lymphoid precursors results in development of plasmacytoid DCs, which are found in the T-cell zones of lymphoid organs and in the thymus and blood. Figure 1.3 describes human DCs subsets with specific sets of surface markers and functions.
1.2.3.3 Functional differences between different subsets of DCs

DCs may develop from a myeloid or lymphoid lineage. The myeloid pathway of differentiation gives rise to DCs that home to peripheral tissues to ingest and process exogenous Ags. This step is prior to migration of DCs to secondary lymphoid tissues and presenting Ags to naïve T cells for activation. Thymic DCs, on the other hand, perform a very different function by being involved in the presentation of self-antigens to developing thymocytes and subsequent deletion of autoreactive T cells. (Ardavin C et al., 1993). This implies that the existence of alternative developmental pathways is in accordance with the different functions assigned to DCs in different tissues.
1.2.4  Role of DCs in control of immunity

Functions of DCs in immune system can be summarized as:

- **Sentinels in vivo:** DCs are distributed *in situ* to optimize the prime function of capturing Ag and migration into lymphoid organs to optimize clonal selection of rare CD4+ and CD8+ T cells.
- **Initiators of immune responses:** DCs play crucial role in stimulation of quiescent, naive and memory, B and T lymphocytes.
- **Potency in stimulating T cells:** Even small numbers of DCs have capability to capture and process low levels of Ags to induce strong T-cell responses.
- **Inducers of tolerance:** DCs are important in process of deletion of self-reactive thymocytes and anergy of mature T cells.

1.2.5  Life history of DCs

The crucial role of DCs in initiation and regulation of T-cell immunity becomes highlighted by presenting a solution to the challenges faced by body in generation of an immune response. These challenges include identification and recognition of peptides from infected cells located anywhere in the body by circulating T-cells, very less amounts of specific Ag-MHC complexes expressed on tumors and infected cells (100 or less/cell) and recognition by rare T-cell clones (1/100,000 or less) through TCR. Primary role of DCs includes capturing Ags from periphery, processing them, display of large amounts of MHC-peptide complexes at their surface, up-regulation of co-stimulatory molecules and migration to secondary lymphoid organs where they activate Ag-specific T cells and mount the appropriate immune response. The life cycle of DCs is summarized in Figure 1.4.
Figure 1.4 - Life cycle of DCs via afferent and efferent limbs of immunity. Ags are captured by DCs located in peripheral tissues and processed to form MHC-peptide complexes. These immature DCs are derived successively from proliferating progenitors and non-proliferating precursors. DCs begin to mature, expressing molecules with T-cells stimulatory properties. Both B and T cells can cluster with DCs resulting in secretion of cytokines and further leaving the T-cell area. B blasts move to the lining of the intestine, the bone marrow and other parts of the lymphoid tissue with some becoming antibody-secreting plasma cells. T blasts leave the blood at the original site of Ag deposition, recognizing changes in the inflamed blood vessels and responding vigorously to cells that are presenting Ag.

DCs display diverse cellular functionality at each stage of their life cycle with the expression of unique set of phenotype markers and secreted factors (Stockwin LH et al., 2000). DCs are characterized by distinct set of cellular markers expressed during various stages, such as migration of precursors from bone marrow to circulation and then to tissues, maturation and activation of DCs (Figure 1.5).
**Figure 1.5 - Subdivision of DCs life history.** Transition between various phases of life cycle of DCs is mediated by diverse signals and is accompanied by changes in expression patterns of many surface markers and secreted factors (cytokines/chemokines).

**1.2.5.1 Trafficking of DCs**

DCs are recognized as migratory cells with potential of trafficking from one site to another, while performing specific functions at each site (Sallusto F, Lanzavecchia A, 2000). DCs originate from hematopoietic stem cells in the bone marrow and the progenitors migrate via blood stream, homing to peripheral tissues. During this process, they encounter several essential growth factors such as GM-CSF, IL-4, IL-15, TNF-α, TGF-β and IL-3, which determine the fate of progenitors to differentiate into “resident immature DCs” but are unable to stimulate T cells (Mohamadzadeh M, Luftig R, 2004).

This extravasation of DCs from the blood to peripheral tissue and the movement from peripheral tissue into lymphoid tissue for maturation requires chemoattractants called “chemokines”. Chemokines are peptide activators of G protein-coupled receptors expressed on leukocytes that regulate recruitment of inflammatory cells into the site of inflammation (Kunkel SL, 1999).
Chapter 1

The ability of DCs to respond to pro-inflammatory and lymphoid chemokine gradients is presumably linked to their maturation state. This ability is based on the fact that as DCs mature they slowly lose the responsiveness to inflammatory chemokines and gain responsiveness to lymphoid chemokines. Immature DCs express both CC and CXC chemokine receptors (CCR and CXCR), such as CCR1, CCR2, CCR5, and CXCR1 and respond to inflammatory chemokines such as Macrophage Inflammatory Protein-1α (MIP-1α), Monocyte Chemotactic Protein-1 (MCP-1) and Regulated on Activation Normal T cell Expressed and Secreted (RANTES) (Dieu-Nosjean MC et al., 1999). DCs mature in response to a variety of danger signals and in doing so they switch the usage and expression of chemokine receptors on their surface from inflammatory to lymphoid homing receptors. Figure 1.6 summarizes the phenotypic and cytokine secretion profile of DCs during various stages of migration and maturation.

(Lipscomb MF, Masten BJ, Physiol Rev, 2002)

Figure 1.6 - The migratory and maturation pathways of DCs. DCs acquire specific abilities at each site during migration and perform specific functions in a stepwise fashion. pDC, precursor DC; iDC, immature DC; mDC, mature DC.
During maturation, DCs downregulate the expression of CCR1, CCR5, and CXCR1 and upregulate the expression of CXCR4, CCR4 and in particular CCR7, a chemokine receptor that responds to Secondary Lymphoid tissue Chemokine (SLC) and Epstein-Barr virus-induced ligand chemokine (ELC) (Sallusto F et al., 1999). The inflammatory chemokines produced by maturing DCs function in both autocrine and paracrine modes to regulate DC trafficking. In an autocrine mode, they initially stimulate, then downregulate, cognate receptors allowing the DC to respond to other chemoattractants. In a paracrine mode, DCs sustain the inflammatory process by recruiting more monocytes, immature DCs and other inflammatory cells to the site of Ag.

**1.2.5.2 Ag capturing and processing by DCs**

Circulating precursor DCs enter peripheral tissues as “immature DCs” where they capture microbial or viral Ags. Although at this stage DCs lack the requisite accessory set of signals for T-cell activation, such as MHC-I/II, CD40, CD80 and CD86. But they are extremely well equipped to capture Ags; a key event in the induction of immunity required to induce full maturation and mobilization of DCs (Figure 1.5). Immature DCs are weak T-cell stimulators, have few MHCs and accessory-molecules, but express many Ag-capturing Fcγ and Fcε receptors. This phenotype changes dramatically within a day and the cells undergo extensive transformation, where Ag-capturing devices disappear and T-cell stimulatory functions increase.

Interstitial DCs and LCs are found at sites that interface with the external environment, i.e., mucosal surfaces and in the skin, highlighting the sentinel function of DCs. Humans have about $10^9$ epidermal LCs, the immature dendritic cells of the skin that are located above the basal layer of proliferating keratinocytes (Banchereau J, Steinman RM, 1998). In peripheral tissues, immature DCs have the ability to migrate towards inflammatory foci where they take up and process available Ags and then emigrate through the lymphatics to draining lymph nodes. Within 1-2 h after activation, phagocytic ability of DCs is readily increased. Then they progressively reduce this capacity during the late
stages of maturation, which acts as a control switch for down-regulating phagocytosis by controlling the extent of Ag internalization (Rescigno M et al., 1999).

DCs possess an array of mechanisms for Ag uptake:

- They can take up particles and microbes by phagocytosis (Reis e Sousa C et al., 1993).
- DCs also exhibit formation of large pinocytic vesicles in which extracellular fluid and solutes are sampled, a process called macropinocytosis (Sallusto F et al., 1995).
- They express receptors that mediate adsorptive endocytosis. Receptors involved in endocytosis can be divided into two classes by their capacity to bind pathogen-associated molecular patterns (PAMP) either directly or indirectly by binding complexes of PAMP and their receptors. These include Fcγ and Fcε receptors (Sallusto F, Lanzavecchia A, 1994), C-type lectin receptors like the macrophage mannose receptor (Sallusto F et al., 1995), DEC-205 (Jiang W et al., 1995) and the new family of ‘danger’ receptors called TLR (toll-like receptors). The transducing receptor for LPS has been identified in TLR-2 and 4. The TLR family of proteins has been described as key players in triggering innate defenses against bacterial and fungal invaders (Ko-Jiunn Liu, 2006).

DCs are considered better APCs on account of proficient Ag uptake pathways. The processes of macropinocytosis and receptor-mediated Ag uptake enable Ag-presentation in DCs so efficient that even the picomolar and nanomolar concentrations of Ag are sufficient, much less than the micromolar levels typically employed by other APCs. However, once the DC has captured an Ag, which also can provide a signal for DCs to mature, its skills to capture Ags rapidly decline, and the assembling of Ag-MHC class II complexes starts.

Thereafter, the Ag enters the endocytic pathway of the cell. TCRs on T lymphocytes recognize fragments of Ags bound to molecules of major histocompatibility complex (MHC) on the surface of DCs. Unlike macrophages, where most of the protein substrate is directed to the lysosomes (an organelle with only few MHC class II molecules where the Ag is fully digested into amino acids), DCs are able to produce large amounts of
MHC class II-peptide complexes in specialized, MHC class II-rich compartments (MIICs) abundant in immature DCs (Nijman HW et al., 1995). During maturation, DCs respond to inflammatory cytokines and microbial products by converting MIICs into non-lysosomal vesicles and discharging their MHC-peptide complexes to the surface (feature of mature T-cell stimulatory DCs). Upon getting the requisite maturation stimulus or signal, fragments of Ag are loaded onto class II molecules and these complexes are sent to the cell surface, where they remain stable for days (Pierre P et al., 1997). To stimulate cytotoxic killer cells, DCs have to present endogenous antigenic peptides complexed with MHC class I molecules to CD8+ Tc cells. The infecting virus uses the cell’s machinery to synthesize viral proteins, which are degraded into peptides and bind to class-I molecules in the endoplasmic reticulum. The peptide-loaded MHC class I complexes travel to the cell surface where they are displayed for scrutiny by T cells (Brossart P, Bevan MJ, 1997). On the other hand MHC-II-antigenic complexes are presented to CD4+ Th cells for generation of effective immune-regulatory responses.

1.2.5.3 Maturation of DCs, Ag presentation, T cell interaction and activation

Once processed, Ag is expressed on cellular surface of DCs along with MHC complexes. DCs emigrate through the lymphatics to draining lymph nodes. There they home to T cell-rich areas in secondary lymphoid organs and interact with T cells to initiate an immune response. During the process of Ag processing and migration to secondary lymphoid organs, DCs acquire a matured state with reduced Ag-capture capacity and high expression of accessory molecules that interact with receptors on T-cells to enhance adhesion and signaling (co-stimulation); such as MHC-I/II, CD40, CD86 and CD80 (Figure 1.5) and can readily prime T cells. Owing to the stellate appearance with cytoplasmic processes, DC has a high surface area permitting intimate contact with a large number of surrounding cells. In vitro DCs result in formation of large spherical aggregates with T-cells and experimentally, only one mature DC is required to stimulate 100-3000 T cells. In vivo, immunity develops in lymphoid organs, where the DC-T-cell interaction can be seen for all major classes of T-cell ligands (Ingulli E et al., 1997). DCs
form clusters with Ag-specific T cells, creating a microenvironment in which immunity can develop.

DCs present to be efficient APCs, as reflected by 10–100 times higher expression of MHC products and MHC–peptide complexes than any other APCs like B cells and monocytes to interact with T cell receptor (TCR). This interaction acts as "signal-1" in DC-activation (Inaba K et al., 1997). As shown in Figure 1.7 DCs also express high surface density of many accessory/co-stimulatory molecules that interact with receptors on T cells to enhance adhesion and signaling (co-stimulation), for example LFA-3/CD58, ICAM-1/CD54, B7-2/CD86, B7-1/CD80, Bp55/CD40. Binding of these co-stimulatory molecules to specific ligands on T-cells are essential for effective activation of T-lymphocytes and acts as "signal-2" for activation of DCs (Stockwin LH et al., 2000, Caux C et al., 1994, Inaba K et al., 1994). B7-1/CD80 and B7-2/CD86 on DCs bind to CD28 molecules on T-cells. Failing this at the time of Ag recognition by TCR, an alternative T lymphocyte function namely 'anergy' may start. Another CD80/86 ligand, CTLA-4 is also induced on activated T-cells contributing to a negative regulatory signal (Kleijmeer MJ et al., 1995, Inaba K, Steinman R, 1986).

Mature DCs resist the suppressive effects of IL-10, but synthesize high levels of IL-12 that enhances both innate (NK cells) and acquired (B and T cells) immunity (Cella M et al., 1996). Secretion or lack of secretion of interleukin-12 (IL-12) by DCs (signal 3) is very important in the final differentiation of CD4+ T cells into type 1 or type 2 effector T cells, respectively. All these properties (MHC expression, expression of co-stimulatory molecules and secretion of IL-12) are upregulated within a day of exposure to many stresses and dangers, including microbial products (Figure 1.7).
Depending on the conditions, DCs can stimulate the outgrowth and activation of a variety of T cells, which can affect the immune response differently. They can either persuade CTLs, which express the accessory molecule CD8+ and hence interact with MHC class-I bearing DCs to proliferate vigorously (Behrens G et al., 2004). CTLs have the capacity to eliminate virally infected cells and tumor cells. On the other hand, following education by Ag-loaded DCs in secondary lymphoid organs, naïve CD4+ T-helper cells differentiate into memory Th cells, which support the differentiation and expansion of CD8+ CTLs and B cells. In the presence of IL-12, CD4+ T cells turn into interferon-g (IFN-γ)-secreting Th1 cells (Koch F et al., 1996). IFN-γ activates the antimicrobial activities of macrophages and together with IL-12, it promotes the differentiation of T cells into killer cells. So the capacity of DCs to produce IL-12 and Th1 cells will lead ultimately to microbial resistance. With IL-4, however, DCs induce T cells to differentiate into Th2 cells which secrete IL-5 and IL-4. These cytokines activate eosinophils and help B cells to make the appropriate antibodies, respectively.
The cytokine profile secreted by DCs varies with the different stages of DC development and maturation, hence influencing the different effector functions of immature vs. mature DCs (Lanzavecchia A, Sallusto F, 2000). A wide variety of cytokines may be expressed (not necessarily simultaneously) by mature DCs including IL-12, IL-1α, IL-1β, IL-15, IL-18, IFN-α, IFN-β, IFN-γ, IL-4, IL-10, IL-6, IL-17, IL-16, TNF-α, and MIF. The exact cytokine repertoire expressed will depend on the nature of the stimulus, maturation stage of the DC and the existing cytokine microenvironment (Liu YJ et al. 2001). The distinct cytokine patterns released by mature DCs ultimately determine their Th1/Th2 polarizing capacities (Moser M, Murphy KM, 2000).

Summarizing the stages in life cycle of DCs, this key cell population efficiently acts as a bridge between innate and adaptive immune responses.

1.2.6 DCs in clinical immunology

Given their central role in controlling the immune responses, DCs are now identified as logical target cell populations for many clinical situations that involve T cells, such as transplantation, allergy, autoimmune disease, resistance to infection and tumors, immunodeficiency and vaccines. Several checkpoints of DC biology present essentially good targets for pharmacological immune-modulation, which are DC-maturation, migration and homeostasis (Schlichting CL et al., 2005). It has been realized that other than primary interference with lymphocytes biology, most of the immunosuppressive agents directly interfere with immune responses at the earliest stage by targeting key functions of DCs. Thus, DCs have been used as target cell-population to evaluate pharmacological action of various immunomodulating compounds, such as Vitamin-D (Vit D3), Rapamycin, Aspirin, Statins and Cyclosporin A (CsA) (Abe M, Thomson AW, 2003, Hackstein H, Thomson AW, 2004).

In addition, Dendritic Cell (DC) therapy represents a new and promising immunotherapeutic approach for treatment of advanced cancer as well as for prevention of cancer (Boudreau JE et al., 2011). Successful cancers are able to circumvent the immune system by several mechanisms, for example, by inducing apoptosis in DCs so
that they are unable to generate a productive immune response, resulting in defected functions of DCs (Esche C et al., 1999). There has been an increasing interest in understanding the role of DCs in cancer immunosurveillance and immunotherapy by use of DC-based tumor vaccines (Gilboa E et al., 1998).

Dendritic Cell (DC) therapy represents a new and promising immunotherapeutic approach for treatment of advanced cancer as well as for prevention of cancer. Because many immunization approaches probably require antigen uptake by in situ DC (potent APCs), it is reasonable to consider using antigen-loaded DC for vaccination (Mosca PJ et al., 2007). Cancer immunotherapeutic strategies include genetic vaccination with DNA encoding tumor specific genes and activating host DCs in situ to enhance tumor resistance by intratumoral or systemic inoculation of DC-mobilizing cytokines such as GM-CSF or Flt3L. One of the more widely examined techniques involves directly loading host’s DCs with tumor antigens in vitro and then inoculating the DCs back into the tumor-bearing host (Gilboa E, 2007). Numerous studies have shown that murine and human DCs loaded with antigen by many different strategies can be used to activate autologous T cells to proliferate and become antigen-specific cytolytic effectors. Other antigen-loading strategies use apoptotic tumor cells or antigen-pulsed DC-derived exosomes, which are vesicles containing high concentrations of MHC, peptide and costimulatory molecules (Hao S et al., 2007). The important outcome with all of these techniques is that tumor antigens are processed by both the endocytic and proteosomal DC pathways and are capable of stimulating both CD4+ and CD8+ T cells for an overall effective immune response. A number of phase I and phase II clinical trials with DC vaccines have been performed in tumor types, including melanoma, renal cell, breast, lung, colon, neuroendocrine and brain tumors. In general, they have been well tolerated with few toxicities referable to the immunizations. In 2012, Center for Immunotherapy at Roswell Park Cancer Institute (RPCI) has launched a phase I clinical research study of a dendritic cell vaccine (primed with Rapamycin) designed to both eradicate cancer cells and prevent disease relapse.
1.3 Inflammation and cancer

Over the years, inflammation has been recognized as a localized protective reaction of tissues to irritation, injury or infection which is characterized by pain, redness, swelling and sometimes loss of function. There has been a new realization about its causative and supporting role in a wide variety of diseases, including cancer. While acute inflammation is a part of the defense response, chronic inflammation can lead to cancer, diabetes, cardiovascular, pulmonary and neurological diseases.

1.3.1 Inflammation: transition from acute to chronic

Originally defined by Celsus around AD40 as ‘rubor, calor, dolor, tumor’ (redness, heat, pain and swelling), inflammation is generally considered to be an integral part of the host’s immune response to either internal or external environmental stimuli. It is a physiological process in response to tissue damage resulting from microbial pathogen infection, chemical irritation, and/or wounding (Medzhitov R, 2008). Inflammatory responses occur in three distinct phases, each apparently mediated by different mechanisms:

- Acute, transient phase, characterized by local vasodilatation and increased capillary permeability,
- Subacute phase, characterized by infiltration of leukocytes and phagocytic cells to site of inflammation,
- Chronic proliferative phase, in which tissue degeneration and fibrosis occur.

Inflammatory response aims to counteract the insult or damage incurred by various stimuli to the host. This response can be pyrogenic, as indicated by fever. During the early stages of this process, neutrophils are among the first cell populations to migrate to the site of inflammation under the tight regulation of various molecules produced by resident macrophages and mast cells in tissues (Sadik CD et al., 2011). Subsequent with the progress of inflammatory cascade, other types of leukocytes, lymphocytes and inflammatory cells such as macrophages, lymphocytes and plasma cells become activated and get attracted to the inflamed site. This migration occurs under the influence of a
signaling network involving a number of growth factors, cytokines and chemokines (Elenkov IJ et al., 2005). All cells recruited to the inflammatory site contribute to tissue breakdown and are beneficial by strengthening and maintaining the defense against infection.

However, this tissue damage is reversible depending on the intensity of reaction. When acute inflammation or fever is manifested for a shorter period of time, it has a therapeutic consequence. The role of proinflammatory cytokines, chemokines, adhesion molecules and inflammatory enzymes has been linked with chronic inflammation. When inflammation becomes chronic or lasts too long, it can prove harmful to host and may lead to diseases such as cancer. Chronic inflammation has been linked to various steps involved in cancer development, such as cellular transformation, promotion, survival, proliferation, invasion, angiogenesis and metastasis (Figure 1.8).

\[\text{(Aggarwal BB et al., Biochem Pharmacol, 2006)}\]

**Figure 1.8 – Different faces of inflammation and role in tumorigenesis**
Acute inflammation may turn into chronic inflammation if the injurious agent persists for a long time. As a checkpoint for inflammatory processes from lasting too long, multiple mechanisms prevail in the body preventing inflammation to sustain beyond a particular time period (Nathan C, 2002, Maiuri MC et al., 2004). These preventive mechanisms include a shift from antibacterial tissue damage to tissue repair mode. This involves both pro-inflammatory and anti-inflammatory molecules as well as reactive oxygen and nitrogen intermediates, which play a dual role in both promoting and suppressing inflammation. In addition, a rapid programmed clearance of inflammatory cells is also required for resolution of inflammation. Neighboring immune cells such as macrophages, dendritic cells, and phagocytes function efficiently towards clearance mechanisms by inducing apoptosis and phagocytosis (Savill J et al., 2002). The phagocytosis of apoptotic cells itself also promotes an anti-inflammatory response, such as enhancing the production of anti-inflammatory mediators (Fadok VA et al., 1998).

On the other hand, if the resolution of inflammation is dysregulated, cellular response changes to the pattern of chronic inflammation. In chronic inflammation, the inflammatory foci are dominated by lymphocytes, plasma cells, dendritic cells and macrophages with varying morphology (Philip M et al., 2004). Macrophages and other inflammatory cells are activated persistently and generate excessive levels of growth factors, cytokines, reactive oxygen and nitrogen species that may cause DNA damage (Coussens LM, Werb Z, 2001). A microenvironment constituted by all the above elements results in sustained cell proliferation and continuous tissue damage, thus predisposes chronic inflammation to alarming condition such as neoplasia (Balkwill F, Mantovani A, 2001).

1.3.2 Cancer development: an overview

Cancer defines malignant neoplasms characterized by metastatic growth, which may occur in almost every single organ and tissue. This abnormal growth relates to a variety of etiologic factors, such as genomic instability and environmental stress. Cancer is a hyperproliferative disorder that primarily involves transformation in cellular morphology,
dysregulation of apoptosis machinery, uncontrolled cellular proliferation, invasion, angiogenesis and metastasis. Various clinical and epidemiologic studies have suggested a strong association between chronic infection, inflammation and cancer (Shacter E, Weitzman SA, 2002). Only a minority of all cancers are caused by germline mutations, whereas the majority of them (90%) are associated with somatic mutations and environmental factors. Many of the environmental causes of cancer and risk factors are associated with some form of chronic inflammation. Up to 20% of cancers are linked to chronic infections, 30% can be attributed to tobacco smoking and inhaled pollutants (such as silica and asbestos) and 35% can be attributed to dietary factors (20% of cancer burden is linked to obesity) (Aggarwal BB et al., 2009).

Specifically, tumorigenesis is a complex and multistep process in which cancer cells originate from normal cells. This results from the accumulation of mutations, leading to the activation of proto-oncogenes and the inactivation of tumor suppressor genes (Schedin P, Elias A, 2004). In this process of cancer development, carcinogenesis is triggered by multiple genetic alterations which confer specific types of growth advantage to tumor cells. Malignant growth is characterized by several key changes, such as self-sufficiency of growth signals, insensitivity to antigrowth signals, escaping from apoptosis, unregulated proliferation, enhanced angiogenesis and metastasis (Hanahan D, Weinberg RA, 2000). Each of these shifts is accomplished by combined efforts of complex signaling processes. The fate of a cancer cell, however, strongly depends on the generation and establishment of a “supportive” environment, the tumor stroma and the establishment of a bidirectional cross-talk among tumor cells, tissue-resident cells (e.g., fibroblasts) and tumor-infiltrating immune cells (Ruegg C, 2006).

1.3.3 The crosslink between cancer and Inflammation

The classic model of cancer development can be defined by six hallmarks, including uncontrollable growth, immortality and the ability to invade other tissues. Increasing evidence suggests that a seventh characteristic feature should also be included in this model—“inflammation” (Candido J, Hagemann T, 2013, Trinchieri G, 2012) (Figure 1.9).
The crucial role of inflammation in tumorigenesis is now widely accepted and it has become evident that an inflammatory microenvironment is an essential component of all the tumors, including even those in which a direct causal relationship with inflammation is not yet proven (Mantovani A et al., 2008).

Figure 1.9 - The Hallmarks of cancer. Hanahan and Weinberg proposed the classic model to define the six properties characterizing a tumor; unlimited replicative potential, ability to develop blood vessels (angiogenesis), evasion of programmed cell death (apoptosis), self-sufficiency in growth signals, insensitivity to inhibitors of growth, tissue invasion and metastasis. Each of these shifts is complicated and accomplished by combined efforts of various signaling processes. Kim and other’s findings (Kim S et al., Nature, 2009, Mantovani A et al., Nature, 2008, Coussens LM, Werb Z, Nature, 2002) indicated that this model should be revised to include cancer-related inflammation as an additional hallmark.

The link between inflammation and cancer was first postulated in the 19th century based on morphological observations. The functional relationship between inflammation and cancer originated in 1863, when Rudolf Virchow noticed massive infiltration of leucocytes into neoplastic tissues and suggested a connection between inflammation and cancer (David H, 1988). He hypothesized that the “lymphoreticular infiltrate reflected that origin of cancer was at sites of chronic inflammation”. Based on his hypothesis it
was proposed that some class of irritants, along with tissue injury and ensuing inflammation causes enhanced cell-proliferation (Balkwill F, Mantovani A, 2001).

Over last few decades, understanding the biology of inflammatory microenvironment of malignant tissues has supported Virchow's original hypothesis. The links between cancer and inflammation are beginning to have implications for prevention and treatment of the disease. Several different lines of work, ranging from epidemiology to gene-modified mice have highlighted the key aspects of inflammation as a predisposing cause of cancer. Recent data from mouse models of human cancer have established that inflammation, which orchestrates the tumor microenvironment is a critical component of both tumor promotion and progression. Virchow's hypothesis has been revisited by many research groups and there are now ample data to corroborate inflammation-mediated oncogenesis, supporting that chronic inflammatory diseases are frequently associated with increased risk of cancers (Coussens LM, Werb Z, 2002; Block TM et al., 2003; Rosin MR et al., 1994).

Recent literature has highlighted the important role of inflammation in promoting cancer (Hagemann T et al., 2007). Specifically, the gastrointestinal system is the site of a significant proportion of these tumors. Cancers arise in chronically inflamed gastrointestinal tissues and organs while starting in the pharynx and moving distally (Macarthur M et al., 2004). The etiology of the inflammation is variable and can be caused by infective agent such as a virus, bacteria, parasite, or a noninfective irritant, either physical or chemical agent. For example, Epstein-Barr virus (EBV) is the etiological agent responsible for the progression of early dysplastic change into severe dysplasia in nasopharyngeal carcinoma (Gullo C et al., 2008). Hepatitis B (HBV) and C (HCV) viruses account for 80% of cases of hepatocellular carcinoma worldwide (Adrian M Di Bisceglie, 2009; Yao F, Terrault N, 2001) and human papilloma virus (HPV) infection is the leading cause of anogenital cancer (Zur Hausen H, 1991). The gram-negative bacterium \textit{Helicobacter pylori} has been identified as the major etiological factor in gastric adenocarcinoma (Matysiak-Budnik T, Mégraud F, 2006). There are strong associations between alcohol abuse, which leads to inflammation of the liver and
pancreas and cancers of these organs. The risk of colorectal cancer is 10-fold greater if linked with inflammatory bowel disease, such as ulcerative colitis and Crohn's disease (Itzkowitz SH, Yio X, 2004). Moreover, the control of colitis by certain anti-inflammatory agents reduced incidences of colon cancer (Moody GA et al., 1996). It was also suggested in the context of the respiratory system that risk of cancer is positively associated with the severity and duration of inflammatory diseases (Borm PJ, Driscoll K, 1996). Thus, it is apparent that chronic inflammation is a common underlying theme in the development of various malignancies.

1.3.4 The inflammatory “tumor-microenvironment (TMEN)”

Traditionally over past, anticancer research has long been focused on the cancer cell itself. However, more recently significant advances have been made in understanding at molecular level about how the rest of our body influences and interacts with the cancer cell. This is called study of the “microenvironment” - the normal cells and molecules that surround a tumor cell in simple words. The tumor microenvironment (TMEN) is defined as milieu of normal cells, soluble factors, signaling molecules, extracellular matrix and mechanical cues that can promote neoplastic transformation, support tumor growth and invasion, protect the tumor from host immunity, foster therapeutic resistance and provide niches for dormant metastases to thrive (Swartz MA et al., 2012).

In nutshell, the conditions prevailing in TMEN are biased towards tumor development. The intricate communication between the tumor cells and the surrounding cells - the microenvironment - helps in understanding the process of tumor development to a better extent. Progression of normal to benign, benign to malignant, malignant to metastatic is driven not just by what's happening inside the tumor cell solely, but also by what's happening around it in the surrounding environment. The function of the immune system during cancer development is complicated involving extensive reciprocal interactions between genetically altered cells, adaptive and innate immune cells, their soluble mediators and structural components present in the “neoplastic microenvironment” (de Visser KE, Coussens LM, 2006). While the normal cellular microenvironment can inhibit
malignant cell growth by mounting an immune response, the modifications that occur within the tumor microenvironment synergistically support abnormal cell proliferation. Tumors shape and alter their microenvironment accordingly and support the development of both tumor cells and non-malignant cells simultaneously (Whiteside TL, 2008). Inflammatory cells and mediators are infiltrated at very high frequency and are present in the microenvironment of most, probably all tumors, irrespective of causation (Figure 1.10).

Figure 1.10 - Cellular infiltrates within the “tumor microenvironment (TMEN)”. Established cancers consist of a wide array of immune cells that contribute to the tumor stroma of a growing malignancy. Tumors possess infiltrating immune cells of both innate and acquired arms, such as myeloid-derived suppressor cells (MDSCs), macrophages, DCs, mast cells, eosinophils, neutrophils, NK cells and lymphocytes. These cells form a complex regulatory network that fosters tumor growth by creating an environment that enables cancers to evade immune surveillance and destruction. (G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; NO, nitric oxide; ROS, reactive oxygen species).
1.3.5 The paradoxical role of inflammatory cells in cancer development

The studies focused at investigating the link between inflammation and cancers first led to the determination whether the reactive oxygen (ROS) and nitric oxide species (NOS) generated by inflammatory cells (recruited to the inflammatory foci to otherwise kill infectious agents) may cause mutagenic assaults and result in tumor initiation and progression (Okada F, 2002). The carcinogenic characteristics of ROS and NOS have been well acknowledged (Wiseman H, Halliwell B, 1996). Thereafter, the role of inflammatory cell populations in cancer development emerged out.

It has been established now that the development of most of, if not all, cancers from inflammation might be a process mediated by a crosstalk between inflammatory cells, cancer cells and a plethora of various inflammatory markers, such as cytokines, chemokines and enzymes, which altogether constitute an "inflammatory microenvironment" (Lin WW, Karin M, 2007). Although this host response is initially supposed to suppress tumors, it may also facilitate cancer development via multiple signaling pathways.

To drive carcinogenesis and transition from normal cells to malignant tumors, more genetic and epigenetic events are essential rather than a single mutation. Some of these important events are found to be related to chronic inflammation, such as angiogenesis. Also, due to the presence of large number of inflammatory cells, the tumor inflammatory microenvironment readily facilitates the breakage of the basement membrane mediated by degrading enzymes. This process is required for the invasion and migration of tumor cells. In most of the cancers, a wide population of leukocytes and other types of immune cells infiltrate to the developing tumor site in large numbers and establish the tumor inflammatory microenvironment (Coussens LM, Werb Z, 2001). The tumor microenvironment is essentially constituted by both tissue-resident as well recruited innate immune cells (Figure 1.10). Instead of executing the expected anti-tumor immune responses, these infiltrating activated-immune cells inhabiting the inflammatory milieu of TMEN exert paradoxical effects during cancer development. These immune cells are not
able to exhibit their normal immunoprotective mechanisms directed towards anti-tumor immunity (de Visser KE et al., 2006; Smith HA, Kang Y, 2013). Most current data support the notion that the inflammation triggered by tumor-infiltrating host leukocytes and other immune cells does not exert normal immunoprotective mechanisms required for eradication of the evolving cancer (antitumor immunity). On the contrary, these cells act coherently as tumor promoters by excessive and chronic secretion of proinflammatory mediators, such as cytokines, chemokines, MMPs and angiogenic factors which contribute to tumor promotion and progression (Smyth MJ et al., 2004). This inconsistent behavior of inflammatory cells contrary to their prescribed function can be attributed to the loss of delicate balance existing between “antitumor immunity” and “tumor-originated proinflammatory activity” within the tumor microenvironment niche (Figure 1.11). Due to prevalence of an inflammatory environment by continuous secretion of cytokines/chemokines, the balance in this situation is diverted more towards tumor development, overpowering the capability of inflammatory cells to project “anti-tumor immunity” (Schetter AJ et al., 2010). Further, accumulated mutations in epithelial cells lead to dysregulation of their growth and migration. This functions as a prompt signal to recruit more and more of leukocytes and inflammatory cells into TMEN, which further support the tumor growth. In addition, tumor cells may also produce cytokines and chemokines to attract immune cells towards TMEN to facilitate cancer development (Lin EY, Pollard JW, 2004).

The mutagenic potential of inflammation is substantiated by microenvironment with chronic inflammation. Macrophages, together with other leukocytes, generate high levels of reactive oxygen and nitrogen species to fight infection. However, in presence of continuous tissue damage and cellular proliferation, the persistence of these infection-fighting agents is deleterious via production of mutagenic agents. This effect is particularly mediated by peroxynitrite, which react with DNA and cause mutations in proliferating epithelial and stroma cells (Maeda H, Akaike H, 1998).
Pro-tumor activities in-turn depend upon different mediators that are released by host inflammatory cells and cancer cells. Under the influence of pro-inflammatory cytokines and chemokines, host-mediated immune mechanisms against tumor development become considerably weaker as compared to tumor-mediated immunosuppression. This results in the escape of tumor cells from the normal immune surveillance mechanisms of body and a sustained release of inflammatory mediators further favoring uncontrolled tumor growth (Hadden JW, 2003). However, when host-mediated antitumor immunity is stronger than tumor-mediated immunosuppressive activity, tumor cells are eliminated. On the other side, the net outcome of a persistent inflammatory microenvironment is an overall enhanced tumor promotion, accelerated tumor progression, invasion of the
surrounding tissues, angiogenesis and often metastasis (Karin M, 2006; Karin M, Greten FR, 2005) and accumulation of immune and various other types of tumor-associated host cells (such as fibroblasts and endothelial cells) (Kim R et al., 2006). These diverse cells communicate with each other by means of either direct contact or cytokine and chemokine production which act in autocrine and paracrine manners to control and shape the tumor growth. The critical factor that dictates in which direction the balance will shift and whether tumor-promoting inflammation or antitumor immunity will ensue, depends upon the expression of various immune mediators and modulators as well as the abundance and activation state of different effector cell types present in the tumor microenvironment. Ultimately, the direction of balance is decided upon secreted levels of various pro-inflammatory markers by inflammatory cells in TMEN.

1.3.6 Cytokines and chemokines - key players in cancer-related inflammation

The immune response to tumors is constituted by a variety of cytokines and chemokines produced by tumor cells as well as host and recruited immune and stromal cells. Cytokines, chemokines, and growth factors play the leading role in the crosstalk existing between cancer cells, blood vessel and infiltrating inflammatory leukocytes in tumor microenvironment (Ben-Baruch A, 2006). The presence of a plethora of inflammatory components secreted by immune/inflammatory as well as cancer cells in the microenvironment of most neoplastic tissues most frequently results in enhanced angiogenesis, resistance to hormones (in hormone dependent tumors) and inhibition of adaptive anti-tumor immunity (Figure 1.12)(Germano G et al., 2008). Tumor cells survival, proliferation and eventually invasion and metastasis are all regulated by inflammatory mediators present at the tumor site. Broadly, cytokines and chemokines are the key components of an intensive dialog promoting angiogenesis, metastasis and subversion of adaptive immunity and changing response to hormones and chemotherapeutic agents. Recent findings suggest that oncogenes representative of different molecular classes orchestrate the production of inflammatory mediators (Balkwill F, Mantovani A 2001). Inflammatory cytokines have also been reported to facilitate the spectrum of tumor development owing to their paradoxical behaviour. TNF-
α, IL-1 and IL-6 have been identified as the primary inflammatory cytokines implicated in tumor associated inflammatory conditions (Dinarello CA, 2006).

![Figure 1.12 - Role of cytokines/chemokines in the crosstalk between tumor cells, blood vessels and infiltrating leukocytes.](image)

(Germano G et al., Cytokine, 2008)

Recently, there has been a remarkable progress in identification of pro-inflammatory gene products that mediate a critical role in tumor development by suppression of apoptosis, proliferation, angiogenesis, invasion and metastasis (Aggarwal BB et al., 2006). Cytokines; TNF and members of its superfamily, IL-1α, IL-1β, IL-6, IL-8, IL-18, chemokines, MMP-9, VEGF, COX-2 and 5-LOX are among the key players. Interestingly, the expression of all these genes are mainly regulated by the transcription factor NF-kB, which is found to be constitutively active and overexpressed in most tumors and is induced by carcinogens (such as cigarette smoke), tumor promoters, carcinogenic viral proteins, chemotherapeutic agents and γ-irradiation (Karin M et al., 2002; Wenshu Chen et al., 2011; Okamoto T et al., 2007; Magne N et al., 2006). Cytokines, including TNF-α, IL-1-β, IL-6, growth factors, and differentiation factors (colony-stimulating factors) are either secreted or membrane-bound molecules that play a major regulatory role in the growth, differentiation or activation of immune cells.
Cytokine signaling could contribute to the progression of tumors in two aspects: the stimulation of cell growth and differentiation and the inhibition of apoptosis of altered cells at the inflammatory site (Pollard JW, 2004; Hudson JD et al., 1999). Based on the connecting link between cancer and inflammation, anti-inflammatory agents that suppress excessive production of pro-inflammatory cytokines that are causative factors of tumor growth in the TMEN, should have a potential in both the prevention and treatment of cancer. Figure 1.13 represents multiple mechanisms involved in development of inflammation related cancer and the effector role of inflammatory mediators.

Figure 1.13 - Summary of mechanisms for the involvement of inflammation in cancer development. Tumor promotion indicates the process during which initiated cells develop into benign lesions. Tumor progression defines the process during which benign tumors progress to malignant carcinomas. Arrows represent the potential targets for anticancer therapy.

TNF-α

Tumor necrosis factor alpha (TNF-α), also known as “cachectin” is a pleiotropic inflammatory cytokine that plays a central role in inflammation, immune system...
development, apoptosis and lipid metabolism (Aggarwal BB, 2003). Human TNF-α is a non-glycosylated protein of 17 kDa with a length of 157 amino acids, however murine TNF-α is N-glycosylated. Produced not only by activated macrophages but also by tumor cells, TNF-α binds to membrane-bound homotrimeric receptors TNFRI and TNFRII. TNF plays a critical role in inflammatory pathways by acting on both tissue destruction and damage recovery. This maintains the reversibility of microenvironments by stimulating cellular change and tissue remodeling (Balkwill F, 2002; 2006). This cytokine possesses a therapeutic role when secreted by the cells of the immune system. However, when dysregulated and secreted excessively in the circulation, TNF-α can mediate a wide variety of diseases, including cancer.

A wide range of pathogenic stimuli induces TNF-α, which may initiate an inflammatory cascade activating other downstream inflammatory cytokines, chemokines, growth factors and endothelial adhesion factors. TNF-α has a contributing role in all steps involved in tumorigenesis, including cellular transformation, DNA damage, inhibition of DNA repair, promotion, survival, proliferation, invasion, angiogenesis and metastasis (Jaiswal M et al., 2000). A number of studies on TNF-α have demonstrated enhancement of cancer cell invasion as a pro-tumor function under influence of inflammatory cytokines (Kulbe H et al., 2005; 2007). Another important role of TNF-α in linking inflammation to cancer might be its regulation of a network of chemokines (Balkwill F, 2004). These findings have highlighted the necessary background for the development of clinical protocols employing TNF-α antagonists in cancer therapy (Mocellin S et al., 2005).

**Interleukins**

Inflammatory interleukins (including IL-1-β, IL-6, IL-8, and IL-18) have been found to be associated with tumorigenesis, which suggests that inflammation is associated with cancer development. Interleukins are involved in different pathways leading to tumorigenesis and have exhibited important role to play in development of tumor mediated by inflammation (Voronov E et al., 2003). Interleukin-1 (IL-1), a key pro-inflammatory cytokine plays important role in development of tumor assisted with
inflammation. It was shown to be associated with augmentation of an important event in cancer; metastasis, a finding which is related to induction of adhesion molecules in target organs (Giavazzi R et al., 1990). IL-1R1 gene targeted mice have provided unequivocal evidence for the pro-tumor potential of IL-1 (Voronov E et al., 2003). IL-1-β secretion into the tumor milieu also induces several angiogenic factors from tumor and stromal cells that promotes tumor growth (Saijo Y et al., 2002). In addition, strong genetic evidence has linked a pro-inflammatory haplotype at the IL-1 gene locus, comprising agonist and antagonist, to the pathogenesis of gastric carcinoma (El-Omar EM et al., 2001). In a pancreatic islet tumor model, a first wave of myc-driven angiogenesis is induced by the inflammatory cytokine IL-1 (Shchors K et al., 2006). Secretion of IL-1α promotes growth of cervical carcinoma (Woodworth CD et al., 1995).

IL-6 is another key growth-promoting and anti-apoptotic inflammatory cytokine (Naugler WE, Karin M, 2008, Ishihara K, Hirano T, 2002) and is also one of the effector signals of activated NF-κB in the tumor promotion. A significant pro-tumoral role for IL-6 has been demonstrated in multiple myeloma (MM) where both an autocrine loop of IL-6 production as well as a paracrine loop by bone marrow stromal cells have been reported (Klein B et al., 1989). In colon cancer patients, IL-6 serum levels were found to be strongly elevated and positively correlated to tumor load (Dranoff G, 2004). The inhibition of IL-6 production and IL-6 signaling suppresses the growth of colon cancer (Becker C et al., 2004).

Chemokines

Chemokines (chemotactic cytokines) belong to a super-family of pro-inflammatory, activation-inducible cytokines or proteins secreted by cells which have the unique ability to induce directed chemotaxis in nearby responsive cells. Broadly, chemokines can be categorized into CC, CXC, XC, and CX3C based on their relative positions of conserved cysteine residues. In the inflammatory processes, chemokines are generally induced by cytokines. These effector molecules constitute the major soluble regulators that control the directional migration of leukocytes to the site of inflammation. It is now well established that chemokines are involved in the promotion of cancer (Ardestani SK et al., 1999). It has also been reported that some specific chemokines can promote tumor cell
growth, facilitate tumor invasion and metastasis in various cancer types. To be noted, that the balance between chemokines with proangiogenic and angiostatic activities is highly critical in regulating angiogenesis by inducing the expression of MMPs and collagenases, which degrade the basement membrane (Hanahan D, Weinberg RA, 2000). RANTES (Regulated upon Activation, Normal T-cell Expressed and Secreted) is a primary chemokine found to be elevated in prostate cancer and pancreatic adenocarcinoma (Vaday GG et al., 2006, Duell EJ et al., 2006). CXCL-8 (IL-8) is another important chemokine secreted in a paracrine manner, which can recruit inflammatory cells to initiate tumor inflammation and angiogenesis, thus facilitating cancer progression (Sparmann A, Bar-Sagi D, 2004; Qazi BS et al., 2011).

Nitric Oxide (NO)

Nitric oxide (NO) is a signaling molecule with a key function in the pathogenesis of inflammation. Under normal physiological conditions, it contributes to an anti-inflammatory effect. On the other hand, NO is considered as a pro-inflammatory mediator that induces inflammation due to over production in abnormal situations (Sharma JN et al., 2007).

Nuclear Factor-kB (NF-kB) is activated by various inflammatory stimuli and its constitutive activation is routinely observed in accelerating the development of cancer from inflammation (Karin M et al., 2002; Xu W et al., 2002). Inducible Nitric Oxide Synthase (iNOS) an enzyme-catalyzing NO production, was found to be overexpressed in chronic inflammatory diseases and various types of cancer (Chen T et al., 2004). NO is identified as an important regulatory molecule in both inflammation response and cancer development (Hussain SP et al., 2004; Hofseth LJ et al., 2003). iNOS is subjected to induction by proinflammatory cytokines, such as TNF-α and IL-1β and transactivation by NF-kB. Thus it may be a downstream effector of cytokines and NF-kB in linking inflammation to cancer (Li Q, Verma IM, 2002). In the tumor microenvironment of chronic inflammation, the continuous generation of NO may lead to DNA damage, disruption of DNA repair and cancer-prone post-translational modification (Goodman JE et al., 2004). Also, increased NO production might result in p53 activation and carcinogenic p53 mutations (Hofseth LJ et al., 2003). NO may also regulate
angiogenesis, leukocyte adhesion, infiltration and metastasis during cancer development (Rao CV, 2004).

1.3.7 Altered functions of DCs in inflammatory tumor-microenvironment

The cellular constituents of a tumor-microenvironment primarily comprise of immune cells that are normally expected to be otherwise found in secondary lymphoid organs. A number of studies have provided clear evidence of infiltrating large numbers of immune cells in both murine and human tumors (Van Kempen LC et al., 2003). Contrary to expected immune response to a pathogen, immune cells that infiltrate and populate within the tumors are dysregulated and functionally impaired on account of an extensive and dynamic crosstalk with cancer cells. The tumor microenvironment contains innate immune cells (including macrophages, neutrophils, mast cells, myeloid-derived suppressor cells, DCs, and NK cells) and adaptive immune cells (T and B lymphocytes) in addition to the cancer cells and their surrounding stroma (which consists of fibroblasts, endothelial cells, pericytes and mesenchymal cells) (de Visser et al., 2006) (Table 1.2). The most frequently found immune cells within the tumor microenvironment are tumor-associated macrophages (TAMs) and T cells. TAMs mostly promote tumor growth and may be obligatory for angiogenesis, invasion and metastasis (Condeelis J, Pollard JW, 2006). However, there is also evidence that many of the T cell subsets found in solid tumors are involved in tumor promotion, progression or metastasis (Roberts SJ et al., 2007). A disruption of specific cytotoxic mechanisms involved in anti-tumor immunity results in tumor growth and progression.

DCs are key players among immune cell populations accumulated in the tumor microenvironment. Known as professional APCs, their presence is expected to generate anti-tumor immune responses by activating T-cells in lymphoid organs. Although the general thought is that DCs are among the first cells migrating to the tumor site and recognizing tumor cells for the induction of specific antitumor immunity, the picture is modified in the scenario of cancer-microenvironment. Under the influence of smoldering inflammation by excessive production of cytokines/chemokines by surrounding immune cells and tumor cells, DCs interact with malignant cells in a way that they develop specific functional impairments. These compromised DCs with immature phenotype fail
to appropriately prime and activate T cells for anti-tumor response to the same extent as of normal DCs residing within lymphoid organs under nonpathologic conditions (Fricke I, Gabrilovich DI, 2006). Several tumor-derived mediators, such as VEGF, macrophage-CSF (M-CSF), GM-CSF, IL-6, IL-10 and gangliosides have been reported to contribute to the altered differentiation of DCs (Gabrilovich DI et al., 1996; Gabrilovich D, 2004). Low levels of co-stimulatory molecules such as CD40, CD80 and CD86 are expressed on surface of these impaired tumor-associated DCs. Expression of indoleamine 2,3-dioxygenase on DCs degrades the essential amino acid tryptophan that leads to the suppression of T-cell immunity (Munn DH et al., 2002). Tumor-associated DCs also possess defects in antigen-presentation machinery with downregulation of MHC class I and II molecules (Gabrilovich D, 2004). Alteration of DC differentiation hampers their ability to execute effective adaptive immune responses against tumor antigens. Other than important role in antigen presentation and T-cell activation during antitumor immunity, DCs play crucial role in excessive cytokine/chemokine production (Table 1.2) and hence immunosuppression in established tumors (de Visser et al., 2006; Hurwitz AA, Watkins SK, 2012).

<table>
<thead>
<tr>
<th>Cell Types</th>
<th>Antitumor</th>
<th>Tumor-Promoting</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophages, dendritic cells</td>
<td>Antigen presentation; production of cytokines (IL-12 and type I IFN)</td>
<td>Immunosuppression; production of cytokines, chemokines, proteases, growth factors, and angiogenic factors</td>
</tr>
<tr>
<td>Mast cells</td>
<td>Production of tumor-specific antibodies?</td>
<td>Production of cytokines and antibodies; activation of mast cells; immunosuppression</td>
</tr>
<tr>
<td>B cells</td>
<td></td>
<td>Production of cytokines?</td>
</tr>
<tr>
<td>CD8(^+) T cells</td>
<td>Direct lysis of cancer cells; production of cytotoxic cytokines</td>
<td>Education of macrophages; production of cytokines; B cell activation</td>
</tr>
<tr>
<td>CD4(^+) Th2 cells</td>
<td></td>
<td>Production of cytokines</td>
</tr>
<tr>
<td>CD4(^+) Th1 cells</td>
<td>Help to cytotoxic T lymphocytes (CTLs) in tumor rejection; production of cytokines (IFN(\gamma))</td>
<td>Production of cytokines</td>
</tr>
<tr>
<td>CD4(^+) Th17 cells</td>
<td>Activation of CTLs</td>
<td>Production of cytokines</td>
</tr>
<tr>
<td>CD4(^+) Treg cells</td>
<td>Suppression of inflammation (cytokines and other suppressive mechanisms)</td>
<td>Immunosuppression; production of cytokines</td>
</tr>
<tr>
<td>Natural killer cells</td>
<td>Direct cytotoxicity toward cancer cells; production of cytotoxic cytokines</td>
<td></td>
</tr>
<tr>
<td>Natural killer T cells</td>
<td>Direct cytotoxicity toward cancer cells; production of cytotoxic cytokines</td>
<td></td>
</tr>
<tr>
<td>Neutrophils</td>
<td>Direct cytotoxicity; regulation of CTL responses</td>
<td>Production of cytokines, proteases, and ROS</td>
</tr>
</tbody>
</table>

(Grivennikov SI et al., Cell, 2010)

Table 1.2- Roles of different subtypes of immune and inflammatory cells in antitumor immunity and tumor-promoting inflammation. Dendritic cells, in specific play tumor-promoting functions by increased production of cytokines, growth factors and angiogenic factors.
DCs along with other immune cells in TMEN potentially act as tumor promoters by sustained and uncontrolled secretion of inflammatory cytokines and chemokines (Grivennikov SI et al., 2010). To evade host immunity, tumors use numerous strategies to hinder normal DC differentiation, trafficking, activity, and longevity (Figure 1.14). Recent research suggests the potential role of intratumoral DCs in antitumor immune responses. However, understanding the mechanisms of DC-tumor cell interaction and modulation of their activity and function is still not complete. Being pivotal immune cell population in tumor-microenvironment, it is necessary to investigate the immunobiology of cytokine network of tumor-associated DCs, which seems to possess different regulatory functions at the tumor site by supporting tumor growth (Shurin MR et al., 2006).

(Shurin MR et al., Cancer Metastasis Rev, 2006)

Figure 1.14 - Modulation of the dendritic cells system at the tumor site by tumor-derived cytokines, chemokines and growth factors
1.3.8 Cytokines/chemokines secreted by inflammatory cells in TME - promising targets for anticancer activity

Based on the information summarized, cancer-associated inflammation presents to be a promising and attractive target of future anti-tumor therapies (Rose-John S, Schooltink H, 2007). Primary inflammatory cytokines (TNF, IL-1, IL-6) expressed by infiltrating leukocytes and tumor cells are becoming central target of potential anticancer therapy (Balkwill F et al., 2005). Cytokines are established as key components and orchestrators of the inflammatory microenvironment of tumors. Hence, cytokines secreted by infiltrating leukocytes and tumor cells play pivotal role in cancer-related inflammation and represent a prime target in therapeutic efforts aimed at taming tumor promoting cancer-related inflammation (Croci DO et al., 2007, Lee S, Margolin K, 2011). In past years, major anticancer efforts have concentrated on the destruction/inhibition of tumor cells. Alternative strategies to modulate the host tumor microenvironment offer additional interesting approaches by investigating the effects on cytokines/chemokines. Initial results in this direction are encouraging and justify continuing efforts to be further explored for further understanding of this area (Madhusudan S et al., 2005, Harrison ML et al., 2007).
1.4 Hematopoiesis

"Hematopoiesis" is defined as the physiological process that involves the production of cellular components of blood. This extensive process includes various stages such as formation, development and differentiation of blood constituents. In a hierarchical and stepwise manner, blood components are formed from immature cells present in the Bone Marrow (BM) and then subsequently released into circulating blood and peripheral organs for further maturation steps and/or perform their assigned effector functions (Warr MR et al., 2011).

Blood and the system that forms it, is known as the "hematopoietic system" and consists of many cell types with specialized and unique functions. The continuous production of these distinct cellular components depends directly on the presence of "Hematopoietic Stem Cells" (HSCs), which are known as the ultimate and only source/precursors of all the blood-forming cells. HSCs are the only self-renewing cells capable of life-long production of all lineages of blood cells (Orkin SH, Zon LI, 2008). HSCs are placed on the top in the hierarchy of progenitors that become progressively restricted to single or several lineages (Orkin SH, 2000). HSCs are pluripotent and multipotent stem cells residing in the medulla of the bone (bone marrow) of adult mammals. These cells are present within highly regulated niches consisting of multiple cell types that directly and indirectly regulate HSC localization, self-renewal and differentiation. These cells have the unique ability to give rise to different mature blood cell types of the myeloid (monocytes, macrophages, neutrophils, basophils, eosinophils, erythrocytes, megakaryocytes/platelets, dendritic cells) and lymphoid lineages (T-cells, B-cells, NK-cells) (Figure 1.15). These facts make the blood-system as one of the well-studied physiological processes and a paradigm for understanding tissue stem cells, their biology, involvement in aging, disease, and oncogenesis.

Since mature blood cells are predominantly short lived, stem cells are required throughout lifetime to replenish the pool of multilineage progenitors and the precursors committed to individual hematopoietic lineages. Thus, hematopoiesis is recognized as a continuous ongoing process, although the location of stem cells and the specific cell types
derived from them keeps on changing during embryonic, fetal and early postnatal development (Zon L.I., 1995). Secreted signaling molecules called cytokines modulate and control the survival, proliferation and differentiation of all the blood cell lineages, mediated by defined sets of transcription factors. The process of hematopoiesis involves a complex interplay between the intrinsic genetic processes of blood cells and their surrounding environment (Chotinantakul K., Leeanansaksiri W., 2012). This interaction determines whether HSCs, progenitors and mature blood cells remain quiescent, proliferate, differentiate, self-renew or undergo apoptosis. Subpopulations of quiescent and active adult stem cells co-exist in the same tissue, in specific microenvironment called stem-cell "niches" (Nakamura-Ishizu A., Suda T., 2013).

Figure 1.15 - Hematopoietic and stromal cell differentiation

In developing embryos, blood formation occurs in the aggregates of blood cells in the yolk sac, called blood islands (Palisa J., Yoderb MC., 2001). As development progresses, blood formation occurs in the spleen, liver and lymph nodes. When bone marrow develops, it eventually assumes the task of forming most of the blood cells for the entire organism. However, maturation, activation and some proliferation of lymphoid cells occurs in secondary lymphoid organs (spleen, thymus and lymph nodes). In children, hematopoiesis occurs in the marrow of the long bones such as the femur and tibia. In
adults, it occurs mainly in the pelvis, cranium, vertebrae, and sternum (Chotinantakul K, Lceanansaksiri W, 2012).

1.4.1 Functional characteristics of HSCs

1.4.1.1 Multipotency and self-renewal

In a healthy adult person, approximately $10^{11}$ - $10^{12}$ new blood cells are produced daily and maintain steady state levels in the peripheral circulation. Approximately 200 billion new erythrocytes, 100 billion leukocytes and 100 billion platelets are produced each day to replace those lost through natural aging processes. In response to hematological stress (hypoxia, infection) the numbers of a particular type of blood cell required to meet physiological demands can expand rapidly by tenfold. This remarkable capacity for lineage-specific expansion while maintaining the appropriate balance of blood cell types resides in a hierarchy of hematopoietic stem and progenitor cells that are found mainly in the bone marrow (BM). At the origin of this hierarchy lies a comparatively rare population of ~50 million pluripotent hematopoietic stem cells (HSCs) (Szilvassy SJ, 2003). HSCs in the bone marrow give rise to heterogeneous populations of actively dividing hematopoietic progenitors, which proliferate and differentiate resulting in generation of mature blood cells.

HSCs are defined by their unique ability to replenish all blood cell types (Multipotency) (Kondo M et al., 2002). HSCs are normally quiescent or cycle very slowly. When stimulated to proliferate, they undergo a series of asymmetric cell divisions. Another essential feature of HSCs is the ability to “self-renew”, that is, to make copies with the identical or very similar potential. The continued presence of stem cells is essential for production of many (and often short-lived) mature blood cells. When these cells proliferate, at least some of their daughter cells remain as HSCs, ensuring that the pool of stem cells does not become entirely depleted. However, other daughters of HSCs (myeloid and lymphoid progenitor cells) can commit to any of the alternative differentiation pathways that leads to production of one or more specific types of blood cells, but cannot self-renew (Akala OO, Clarke MF, 2006).
1.4.1.2 Differentiation

Differentiation into committed progenitors and mature cells that fulfill the normal functions required for hematopoietic system is not only a unique HSC property, but, together with the option to self-renew, defines the core function of HSCs. Differentiation of hematopoietic cells is driven and controlled by an intricate and complex network of growth factors and cytokines (Seita J, Weissman IL, 2010). It is established, once they commit to differentiation, HSCs cannot revert back to their self-renewing state. Thus, specific signals provided by growth factors seem to be basic requirement for maintenance of HSCs. This strict regulation may reflect the proliferative potential of HSCs, deregulation of which could easily result in malignant diseases such as leukemia or lymphoma.

1.4.1.3 Mobility

Migration of HSCs occurs at specific times during development (i.e., seeding of fetal liver, spleen and eventually, bone marrow) and under certain conditions (e.g., cytokine-induced mobilization) later in life (Lapidot T et al., 2005). HSCs have a higher potential than other immature blood cells to cross the bone marrow barrier and hence they can travel in the blood from the bone marrow in one bone to another bone. HSC normally reside in the bone marrow but can be forced to migrate into the blood, a process termed “mobilization” used clinically to harvest large number of cells for transplantation. At the same time, homing potential of HSCs to the BM is necessary to optimize cell engraftment (Suárez-Álvarez B et al., 2012). If they settle in the thymus, they will develop into T cells. In the case of fetuses and other extramedullary hematopoiesis, HSCs may also settle in the liver or spleen and develop. This ability is the reason why HSCs may be harvested directly from the blood.

1.4.1.4 Expression of surface markers

While initial studies with HSCs aimed to determine their activity in mixed populations, much progress has been made over past in specifically describing the cells that have HSC
activity. A variety of HSC-specific surface markers have been identified to recognize and isolate HSCs (Mayle A et al., 2013). Initial efforts were focused to study HSC properties based on cell size, density and recognition by lectins (carbohydrate-binding proteins derived largely from plants), but more recent efforts have focused mainly on cell surface protein markers, as defined by monoclonal antibodies which have enabled isolation, purification and enrichment of HSCs (Lin KK, Goodell MA, 2011). For mouse HSCs, these markers primarily include panels of 8 to 14 different monoclonal antibodies that recognize cell surface proteins present on differentiated hematopoietic lineages, such as the red blood cell and macrophage lineages (thus, these markers are collectively referred to as “Lin”), as well as the proteins Sca-1, CD27, CD34, CD38, CD43, CD90.1 (Thy-1.1), CD117 (c-Kit), AA4.1 and MHC class-I, and CD150 (Grant AC et al., 2009). Human HSCs have been defined with respect to staining for Lin, CD34, CD38, CD43, CD45RO, CD45RA, CD59, Thy1/CD90, CD109, CD117, CD133, CD166 and HLA DR (human) (Reitsma MJ et al., 2002)

1.4.2 Hematopoietic growth factors

At the level of each individual stem or progenitor cell, the probability of executing any one of the developmental options, or of dying by apoptosis is tightly regulated by a network of glycoprotein hormones known as the hematopoietic growth factors (HGFs). Hematopoietic growth factors comprise an important group of proteins that predominantly regulate the process of haematopoiesis (Tabbara IA, Robinson BE, 1991). HGFs exhibit a general hierarchical organization in their actions that mirrors the cellular elements of the hematopoietic system. However, there is considerable overlap in target cell populations and some cytokines that were originally thought to act only on lineage-committed progenitor cells or their progeny are now known to have multiple levels of activity. Most HGFs are produced by macrophages, fibroblasts, osteoblasts and endothelial cells that comprise the BM microenvironment. These so-called stromal cells also express adhesion molecules that serve to physically retain stem and progenitor cells within “niches,” thus co-localizing them with factors that regulate the earliest events in their development.
Landmark discovery of erythropoietin initiated the era of growth factors research. The identification of growth factors that stimulate the proliferation and maturation of hematologic cells has been a major advance in the fields of hematology and oncology. In 1906, it was found that when healthy rabbits were injected with serum from anemic animals, it prompted a rapid increase of erythrocytes in the recipients (Carnot P, Deflandre C, 1906). Following research suggested that the responsible humoral substance for increase in erythrocytes was "hemopoietine" and was later named "erythropoietin" (Bahlmann FH et al., 2004). In 1957, Jacobson and colleagues identified kidney as the source of erythropoietin (Jacobson LO et al., 1957). In 1985, the factor was purified and its gene was cloned, thereby making it available for physiological study and therapeutic use (Jacobs K et al., 1985). Subsequently in a similar fashion, humoral substances that support the production of leukocytes (Welte K et al., 1996) and platelets (Kaushansky K, 2003) were identified, cloned and studied. Simultaneously with all these advances in the field of growth factors, a model of blood-cell production was constructed. Hematopoiesis was envisioned as a hierarchical progression of multipotential hematopoietic stem cells that gradually lose one or more developmental options, becoming progenitor cells committed to a single lineage; these progenitors then mature into the corresponding types of peripheral-blood cells.

The hematopoietic growth factors are a group of proteins, including erythropoietin, thrombopoietin, interleukins and colony-stimulating factors which are potent regulators of blood cell proliferation and development in the bone marrow (Groopman JE et al., 1989). The hematopoietic growth factors are potent regulators of blood-cell proliferation and developmental process. Lineage-specific growth factors are required for the survival and proliferation of hematopoietic cells at all the stages of development. Of the factors that affect multipotential cells, steel factor, Fms-like tyrosine kinase 3 (FLT3) ligand, granulocyte–macrophage colony-stimulating factor (GM-CSF), IL-2, IL-3 and IL-7 are among the best characterized (Kaushansky K, 2006). Each of these proteins supports the survival and proliferation of a number of distinct target cells.

A number of recently identified cytokines have been implicated in the development of blood cells and their functional activation. These cytokines have extensive clinical
applications in oncology and other disease areas. Since the regulatory approval and commercial launch of the first recombinant human (rHu) HGFs in the early-mid-1990s, several such agents have been administered to millions of patients. Much attention has been focused on the late-acting cytokines such as erythropoietin (EPO), granulocyte colony-stimulating factor (GCSF) and Thrombopoietin (TPO) because these have proven to be most useful for the treatment of different cytopenias. Clinical development of r-HuSCF (ancestim, STEMGEN®) focused on its use in combination with r-HuG-CSF (filgrastim, NEUPOGEN®) to optimally mobilize hematopoietic stem and progenitor cells for transplantation after myeloablative therapy in cancer patients (Lacerna L et al., 2000). The two that have been studied most extensively clinically are G-CSF and GM-CSF with applications in bone marrow insufficiency (primary or secondary to chemotherapy), myelodysplastic syndromes, AIDS and bone marrow transplantation (Offner FC, Van Hove WZ, 1989). Filgrastim (Hu-GCSF) and Neupogen (Hu-GM-CSF) are used to treat neutropenia (a low number of neutrophils) resulting from chemotherapy or bone marrow transplantation by stimulating the bone marrow to increase production of neutrophils (Hassan BA et al., 2009).

1.4.3 Colony Forming Unit (CFU) progenitors in hematopoiesis

Differentiation of HSCs to lineage specific primitive progenitor cells like myeloid stem cells (IL-3 and GMCSF) or lymphoid stem cells (IL-3) permits proliferation of the committed progenitor cells into other categories of major marrow cells. During the process of hematopoiesis, CFU progenitors emerge as precursors which are committed to a specific lineage. Presence of definite set of cytokines/growth factors monitors their development and differentiation into different lineages. The various progenitor cells are identified by the type of "colony" they form. In culture media the progenitor cells are defined as CFUs (Colony Forming Units). The earliest detectable hematopoietic progenitor cell that gives rise to granulocytes, erythroblasts, monocytes and megakaryocytes is termed CFU-GEMM (Colony-Forming Unit Granulocyte Erythrocyte Monocyte Macrophage). Figure 1.16 represents general model of hematopoiesis in a step-wise manner with generation of CFU-progenitors as intermediate cell populations.
1.4.3.1 **Primitive progenitor cells** – Blood-cell development progresses from a multipotent hematopoietic stem cell (HSC), which can undergo either self-renewal or differentiation into a multilineage committed progenitor cell. HSC differentiate in presence of a defined set of growth factors into a lymphoid stem cell/common lymphoid progenitor or myeloid stem cell/common myeloid progenitor.

1.4.3.2 **Committed precursor cells** - These cells then give rise to more-differentiated progenitors in presence of specific combination of cytokines/growth factors. At this stage, Colony Forming Units (CFU) are formed, which are capable of differentiating further into committed cell lineages. There are various kinds of colony-forming units:

- **a)** CFU-E (Colony Forming Unit-Erythrocytes) results from BFU-E (Blasts Forming Unit-Erythrocytes) and leads to generation of RBCs in presence of IL-3, GM-CSF and Epo.
- **b)** CFU-Mk (Colony Forming Unit-Megakaryocytes) – Forms platelets/thrombocytes in the presence of IL-3, GMCSF, IL-6 and TPO.
- **c)** CFU-GM (Colony Forming Unit-Granulocytes/Macrophages) – Bifurcates into CFU-G and CFU-M in presence of IL-3, GM-CSF and G-CSF. Further, CFU-G develops into neutrophils and CFU-M develops into monocytes, macrophages and dendritic cells.
- **d)** CFU-Eo (Colony Forming Unit-Eosinophils) – results into formation of eosinophils in presence of IL-3, IL-4, IL-5 and GM-CSF.
- **e)** CFU-Bas (Colony Forming Unit-Basophils) – develops into basophils in presence of IL-3, IL-4 and GM-CSF.
- **f)** CFU-Mast (Colony Forming Unit-mast cells) – leads to formation of mast cells in presence of IL-3 and SCF.

1.4.3.3 **Lineage-committed cells** - Ultimately, these cells then give rise to “uni-lineage” committed progenitors leading to formation of effector cells in blood. In the lymphoid lineage, antibody secreting B cells, NK cells and T cells are formed. In myeloid lineage, erythrocytes, platelets, neutrophils, macrophages, DCs, eosinophils, basophils and mast cells are the end products. All throughout, the entire process of differentiation is mediated by specific cytokines and growth factors that support the survival, proliferation or differentiation of each type of cell (Reya T, 2003).
Figure 1.16 - Model of hematopoiesis from pluripotent Hematopoietic Stem Cell (HSC)
1.4.4 Functional assays to characterize stem and progenitor cells

In order to characterize hematopoietic stem and progenitor cells in vitro and in vivo, a number of assays have been reported. HSCs have the unique property of “colony” formation in semisolid medium, which has been well exploited in a variety of functional assays to investigate their properties and role in hematopoiesis. CFU assays are powerful tools for evaluating stem cell and progenitor activities in various hematopoietic tissues, during development as well as in the adult animal and in cell populations manipulated ex vivo (Miller CL et al., 2008). In vitro assays for primitive hematopoietic cells (colony-forming units-blast, cobblestone area-forming cells, long-term culture-initiating cells; LTC-IC have been established which demonstrate the proliferative and differentiation capacities of these populations as shown in Figure 1.17 (Bock TA, 1997).

1.4.4.1 Colony-forming unit spleen (CFU-S) assay - Conventional in vivo assays to study HSCs include Till and McCulloch’s classical spleen colony forming (CFU-S) assay (Siminovitch L et al., 1963). CFU-S is considered as the basis of an in vivo colony formation, which depends on the ability of infused bone marrow cells to give rise to clones of maturing hematopoietic cells in the spleens of irradiated mice after 8 to 12 days. It was used extensively in early studies, but is now considered to measure more mature progenitor rather than stem cells (Eaves C et al., 1997).

1.4.4.2 Long-term competitive reconstitution assay - Reconstitution assays involved preconditioning of mice by lethal irradiation to accept new HSCs and injection with purified HSCs or mixed populations containing HSCs, which will repopulate the whole hematopoietic systems of the host mice for the life of the animal (Szilvassy SJ et al., 1990). Long-term presence of donor-derived cells in a reconstituted host is specifically evaluated by studying donor and host specific marker expression, such as CD45.

1.4.4.3 Long-term culture initiating cell (LTC-IC) assay - founded on the bone marrow long-term culture (LTC) system, these assays measures primitive hematopoietic stem cells (termed LTC-IC) based on their capacity to produce myeloid progeny for at least 5
weeks. These assays depend on the capacity of single cells to produce myeloid cells while being attached to an underlying adherent and pre-established supportive stromal layer of either whole bone marrow or a permanent murine cell line (Van Os RP et al., 2008). LTC-IC assays provide as system to quantitate primitive hematopoietic progenitors with a good correlation in vivo, but do not run an accurate measure of totipotent HSCs.

1.4.4.4 Cobblestone area forming cell (CAFC) assay - This assay is a cell culture-based empirical assay in which HSCs are plated onto a confluent culture of stromal feeder layer. A fraction of cells creep in-between the gaps and eventually settle between the stromal cells and the substratum or trapped in the cellular processes between the stromal cells to give rise to “cobblestone-like structures”. Frequencies of different hematopoietic cell subsets growing as cobblestone-area forming cells underneath the stromal layer are measured after 5-7 weeks (de Haan G, Ploemacher R, 2002).

Figure 1.17 – Conventional assays used to detect hematopoietic stem cells

(http://faculty.ksu.edu.sa/17212/lectures/CLS%20541/HSC%20assays-CLS%20541-lecture)
1.4.5 Drug induced hematotoxicity

There is always a balance between the constant production of new cells and the loss of existing healthy, mature and functional cells that maintain the pool of peripheral blood counts. Due to its rapid turnover, the haemopoietic tissue has the integral capacity to respond instantly to an increased demand for production of mature cells (for example, following blood loss or infection), and this response can be maintained for prolonged periods of time. This rapid rate of renewal also makes the hematopoietic system an extremely sensitive target for xenobiotic (a chemical which is found in an organism but which is not normally produced or expected to be present in it) toxicity (Deldar A, 1994). The rapidly dividing bone marrow progenitors are constantly destroyed by substances such as antineoplastics, microbial toxins and ionizing radiation. A single exposure can result in acute and reversible neutropenia or thrombocytopenia 4 to 20 days later. A rapid repopulation of the progenitor compartment precedes the recovery of peripheral counts by several days. Blood and hematopoietic tissue rank with liver and kidney as most sensitive target organs worthy of careful scrutiny in preclinical and clinical safety evaluations (Bloom JC, 1993).

"Hematotoxicity" is the study of blood and blood-forming tissues as a target organ for drugs, chemicals in the environment or workplace and factors such as stress, exercise and ionizing radiation. Benzene has been identified as the major human leukemogen, exposure to which results in various hematological malignancies (Hirabayashi Y et al., 2004). Like other rapidly dividing tissues, such as intestine and gonads, bone marrow is highly sensitive to several classes of drugs and non-therapeutic chemicals. Hematological toxicity (acute cytopenia or bone marrow suppression) is a frequently encountered side effect of cytotoxic chemotherapy for cancer (Bruce WR et al., 1966). In the process of rapidly destroying proliferating cancer cells, chemotherapy also induces damaging side effects simultaneously to other normal, non-cancerous and rapidly dividing cells, such as hematopoietic and other immune cells in the blood and bone marrow. (Kuhn JG, 2002). Thus, conventional antineoplastics, high-dose therapies and drug combinations of most anticancer agents/cytotoxic oncology drugs lead to severe bone marrow (BM) suppression as the most common dose-limiting toxicity in human (Crawford J et al.,
2004). This results in risk of serious infection and hemorrhagic complications. Myelosuppression leads to an overall compromised immune system making the host prone to various infections. Hence, for virtually all the anti-cancer treatments, the differential effect of the drug on cancer cells and normal cells will ideally define the therapeutic index. Impaired hematopoiesis results in low neutrophil count; neutropenia (most common form of myelosuppression induced by anticancer drugs), low platelets; thrombocytopenia and low RBCs; anemia.

Underlying this toxicity is usually a transient, drug-induced reduction in the number or functional bone marrow progenitors in the affected blood cell lineage. This reduction is followed by a recovery that precedes the nadir (the lowest level of a blood cell count while a patient is undergoing chemotherapy) and recovery, respectively, of peripheral blood cell counts (Lyman GH et al., 2005). This transient drop in functional progenitors can be measured using multiple functional in vitro colony forming assays. Cytopenia often determines a drug’s tolerable dose, use in combination therapy and frequency of administration (Hoagland HC, Gastineau DA, 1996). Therefore, it would be highly advantageous to predict during the process of drug development whether a new antineoplastic agent will be clinically myelosuppressive and if yes, upto what extent. Direct in vitro exposure of hematopoietic cells to myelosuppressive drugs causes almost similar reduction in progenitor survival as it would cause in vivo, which is the basis of modern era of in vitro hematotoxicology (Clarke E et al., 2007).

1.4.6 In vitro Colony-forming cell (CFC) assays

Conventional hematotoxicity testing begins in the preclinical studies and is valuable for monitoring the clinical status of subjects in clinical trials. However, since it is based on investigating mature blood elements and related parameters and not the progenitors/precursor stem cells producing these elements, it is not very much predictive. On the other hand, in vitro assays detecting stem and progenitor cell populations are highly predictive and colony-forming assays using human hematopoietic tissue allow hematotoxicity testing to be performed throughout drug development (Rich IN, 2003,
Clonogenic CFC assays are being widely employed to quantify multipotential progenitors and single lineage restricted progenitors of the erythroid, granulocytic, monocyte-macrophage and megakaryocytic pathways (Deldar A, Stevens CE, 1993). When cultured in a suitable semi-solid matrix, such as methylcellulose supplemented with nutrients, growth factors and cytokines, HSCs or hematopoietic progenitors called colony-forming cells (CFCs) proliferate to form discrete cell clusters or colonies which may be enumerated (Pessina A et al., 2005).

Many hematopoietic progenitors produce colonies in vitro when stimulated with appropriate set of cytokines, hence the name 'colony forming unit' or CFU. In vitro clonal assays provide an environment for hematopoietic cells to proliferate mimicking the in vivo conditions of hematopoiesis. The number of colonies formed is proportional to the number of viable progenitors. The degree of inhibition of colony formation resulting from in vivo or in vitro exposure to drug is used to evaluate the cytotoxicity of a variety of compounds (Parent-Massin D, 2001). The type of blood cells found in each colony determines the name of the specific CFU (Figure 1.18). The precursors of neutrophils and monocytes are called granulocyte-macrophage colony-forming cells (CFU-GM). The erythroid burst-forming units (BFU-E) and megakaryocyte colony-forming units (CFU-Mk) are the counterparts of the CFU-GM in the erythroid and megakaryocytic lineages, respectively. CFU-GM is the most attractive and well-studied progenitor for in vitro hematotoxicology studies because of its role in drug-induced neutropenia, its predictive value and the technical simplicity of the assay (Grever MR, Gneshaber CK, 1997).

In vitro hematotoxicology involves the application of specialized cell culture assays to study adverse effects of xenobiotic exposure on the development of specific hematopoietic cell type of interest (Parchment RE et al., 1998). Now, in vitro models of hematopoiesis are being used increasingly in investigative-hematopathology and in preclinical safety studies on candidate drugs (Negro GD et al., 2001; Ratajczak MZ et al., 1993).
The type of hematotoxicities most frequently and most thoroughly studied in vitro are the acute effect of toxicants on bone marrow progenitors, such as granulocyte-macrophages (CFU-GM), erythroids (CFU-E/BFU-E), megacaryocytes (CFU-Mk) and granulocytes-erythroid-macrophage-megakaryocyte (CFU-GEMM). The extent of drug-induced hematotoxic effect is quantified from the number of surviving progenitors as a function of exposure level under maximally stimulatory cytokine concentrations. Since hematotoxicity can result from direct interference by toxicants with hematopoietic precursors, cytokine production, cytokine receptors, binding presentation at the cell surface and/or secretion, many different protocols have been developed and proposed for in vitro toxicity testing based on the modifications from basic soft agar technique (Bradley TR, Metcalf D, 1966).
CFU/BFU-E assays have been employed to evaluate hematotoxicity on erythropoiesis by various classes of compounds, such as antivirals (Gribaldo L et al., 2000), pesticides (Malerba I et al., 2002), anticancer drugs (Malerba I et al., 2004, Su WC et al., 2000). Similarly, to investigate thrombocytopenic effects, CFU-Mk assays have been used as model systems (Pessina A et al., 2009; Parent-Massin D, Sibiril Y, 2008; Froquet R et al., 2001).

1.4.7 Applications of in vitro hematotoxicology predictive assays

Myelotoxicity is established as one of the major limitations to the use of full doses of antitumor agents (Viano I et al., 1986). The goal in the regulatory setting primarily emphasizes the prediction of two levels of exposure: the highest dose that will not cause a clinically adverse effect and the dose that causes maximally tolerated, reversible perturbations in peripheral blood counts, termed the maximum tolerated dose (MTD). Selection of the starting dose for the phase I trial, which is typically based on the MTD of the most sensitive species, is critical. The dose selected must not be toxic, while at the same time it must be high enough to give the patients therapeutic benefit. Even with the advent of the use of molecular targeting to develop new therapeutic approaches, the majority (50% in the last 15 years) of anticancer drugs still produce myelosuppression as the dose-limiting toxicity (DLT) in humans resulting in impairment of immune functions (Parchment RE, 2000).

CFU assays serve as important in vitro predictive tools in hematology and may be helpful to predict drug exposure levels that will be toxic during phase-I clinical trials. These models are also useful for determining the relative sensitivities of various animal species to hematotoxic effects of drugs and for studying synergistic and antagonistic effects (Du DL et al., 1990). One of the important goals during preclinical drug development is to predict whether a new agent will be clinically toxic to the bone marrow, whether the toxicity will be specific to one cell lineage (lymphocytes, neutrophils, megakaryocytes, or erythrocytes), at what dose or plasma level the drug will be toxic, which model best
predicts the clinical situation, and when the onset and nadir of cytopenia and the onset of recovery will be likely to occur (Figure 1.19).

![Figure 1.19](image)

**Figure 1.19 - Four quantitative parameters that describe neutropenia:** nadir, time to nadir, duration of the nadir and time to recovery. Predicting these parameters for a xenobiotic exposure is the purpose of *in vitro* hematotoxicology.

1.4.8 **CFU-GM assay to predict drug induced neutropenia**

Cytotoxic chemotherapy suppresses the hematopoietic system, impairing host’s protective mechanisms and limiting the doses of chemotherapy that can be tolerated. Neutropenia, the most serious hematologic toxicity is associated with the risk of life-threatening infections as well as chemotherapy dose reductions and delays that may compromise the treatment (Crawford J *et al.*, 2004; Lyman GH, 2006). Since neutropenia is acknowledged as the most serious hematotoxic side effect of anticancer drugs, researchers working in the field of the hematotoxicity and predictive toxicology have primarily focused on CFU-GM assays to investigate adverse effects on neutrophil population. Acute toxicant exposure causes decreases in bone marrow progenitors content that precedes and in large part determines the depth of the neutrophil nadir. Also, experiments with antineoplastic agents in animal models reveal a direct relationship between the reduction in granulocyte-macrophage progenitors (CFU-GM) and the decrease in absolute neutrophil count (ANC) (Parchment RE *et al.*, 1993). An *in vitro-*
vivo correlation was found between the severity of neutropenia in the clinic with pyrazoloacridine and the inhibition of CFU-GM *in vitro* (Parchment RE *et al.*, 1994). Subsequently, *in vitro-in vivo* correlations were found for the camptothecins (Erickson-Miller CL *et al.*, 1997) and anguidine (Parent M, Parchment RE, 1998), indicating a clear relationship between the reduction in CFU-GM and the decrease of the ANC. These results strongly suggested that *in vitro* toxicology tests using CFU-GM should be able to successfully predict the exposure levels of xenobiotics that would cause clinical neutropenia after acute exposure.

An international prevalidation study was conducted to optimize the Standard Operating Procedure (SOP) for detecting myelosuppressive agents by CFU-GM assay and to study a model for predicting (by means of this *in vitro* hematopoietic assay) the acute xenobiotic exposure levels that cause maximum tolerated decreases in ANC (Pessina A *et al.*, 2001). In these studies, murine and human bone marrow progenitors were cultured in presence of various growth factors and MTDs were calculated for six well known anticancer drugs. The prediction model applied in this study was based on prediction of human MTD by adjusting the animal-derived MTD for the differential sensitivity between CFU-GM from animal species and humans (Parchment RE, 1998), according to the following algorithm:

\[
\text{Predicted human MTD} = \text{Actual murine MTD} \times \left( \frac{\text{IC}_{90} \text{ human CFU-GM assay}}{\text{IC}_{90} \text{ murine CFU-GM assay}} \right)
\]

Since pharmacokinetic differences across species may contribute to as much as a fourfold difference in MTD, ‘accurate prediction’ in this model is generally defined as the prediction of a human MTD that lies within fourfold of the actual human MTD. This model offers the advantage of being mechanism naive and would only miss the hematotoxicants that adversely affect myelopoiesis via indirect physiological mechanisms, such as induced release of inhibitory cytokines, inhibited release of stimulatory cytokines or metabolic activation of pro-toxicants.

Further a panel of 20 well-known drugs including antineoplastics, antivirals and pesticides were tested in this predictive model to apply this model to clinical neutropenia.
and calculate the human maximum tolerated dose (MTD) by adjusting a mouse-derived MTD for the differential interspecies sensitivity (Pessina A et al., 2003). Based on these results, CFU-GM assay has been approved by ECVAM (European Centre for the Validation of Alternative Methods) at its 24th meeting in 2006 to predict acute neutropenia in humans as a substitute to using a second species, such as the dog, for this purpose.

"The hematotoxic potential of xenobiotics is determined by the evaluation of the inhibition of CFU-GM growth."

Validated and predictive in vitro haematotoxicity assays have the potential to substantially refine and optimize animal use for hematotoxicity testing and could make estimations for human toxicology studies possible in the preclinical setting. These assays could play a key role in bridging the gap between preclinical toxicology studies in animal models and clinical investigations. In vitro tests could refine the safety margins by reducing toxicological uncertainties due to animal/human extrapolation. This would provide a more rational basis for calculating clinical dosages and for setting human exposure limits.

With anticancer drugs, in vitro hematotoxic studies should be undertaken to identify those compounds that are significantly more toxic to humans than to either dogs or rodents. By identifying such compounds, it would be possible to decrease the risk of a lethal overdose in the first cohort of patients to which they are administrated, a risk that cannot be identified during current preclinical testing strategies. An in vitro assay could highlight the potency difference between humans and the preclinical test species, so that the starting dose in phase I clinical trials could be considerably closer to the MTD, without compromising safety. Thus, not only would phase-I clinical trials be completed more quickly, but fewer patients would be treated with ineffective doses. (Grande T, Bueren JA, 1995). These considerations suggest that validated in vitro tests for hematotoxicity could definitely contribute to a reduction in the number of animals required in preclinical toxicology (Balls M et al., 1995; Curren RD et al., 1995).
1.4.9 Application of bone marrow derived cells based assays in drug discovery and research

The development in the area of biotechnology has enriched our current understanding of the immune system. It is now possible to study, analyze and manipulate biological systems at cellular and molecular level. This advancement has accelerated the efforts of finding new therapeutic and prophylactic measures for various clinical indications.

The process of drug discovery and development is very expensive and time-taking. Moreover, discovery of novel molecules in different therapeutic areas with conventional drug discovery approaches includes screening of large number of molecules using *in vivo* animal models. These efforts directed towards development of a new drug generally follow the routine and generalized process. The early stages of drug discovery, such as target identification and validation involve screening strategies to identify the hit compounds. Secondary assays investigate the activity and *in vitro* pharmaceutical and safety assessments, animal pharmacology and toxicology to optimize lead compounds to drug candidates. These steps mostly depend on the use of engineered cell-lines, animal derived primary cell systems or animal based *in vivo* models. However, the poor predictability of early tests contributes to more than 95% attrition of drugs during early phases of this process (Kola I, Landis J, 2004). Search for more reliable, predictive, refined and robust preclinical models for screening, pharmacology and safety assessment is “need of the hour” to reduce the current rate of attrition of drug candidates. Since clinical trials consume most of the cost and time spent on drug discovery and development, new strategies towards improved predictive value of preclinical evaluations are required.

In modern drug discovery, cell-based assays have been widely employed with routine usage of immortalized secondary cell-lines. In pharmaceutical research, *in vitro* toxicity tests are necessary for assessing the potential toxicity of new chemical entities in the early stages of the developmental process, when no information is available about the metabolism or even the target organ toxicity of the compounds to be tested. An implicit assumption in all of these approaches is that the functional response of the cell provides a
better understanding of both the physiology of the drug target, as well as the pharmacological interaction with novel compounds. Cell-based assays can distinguish between agonists and antagonists, identify allosteric modulators, provide direct information on compounds with regards to cell permeability and stability inside cells and acute cytotoxicity associated with the compounds.

However, classical cell-based assays employing cultured immortalized cell-lines possess phenotypes that differ markedly from those found in vivo in human pathology and have historically been chosen based on their ease of use with prevailing screening technologies. On account of continuous passages, mutations may arise in cell lines leading to alterations in their characteristic features. The dependence on the use of immortalized cells for many years has raised some serious concerns in terms of the clinical relevance of either the target validated, or of the identified lead compound selected for subsequent development. Consequently, there is an emerging interest in usage of primary or stem cells based screening strategies that can be adapted to sophisticated high content screening (HCS). In primary cells, the endogenous target is assumed to be expressed in an environment that more closely mimics and resembles the conditions of human disease. Consequently, novel drugs characterized using these primary cell based systems are presumed to act in a more targeted and predictable fashion, as one might find in the disease, than those characterized in immortalized cells.

In vitro assay systems based on primary hematopoietic precursor cells, such as bone marrow cells, can be developed under specific, sophisticated culture conditions to generate cells of interest. Murine-bone marrow derived Dendritic Cells (BMDC) and CFU assays can be used to understand anticancer potential of new compounds, as indicated by their anti-inflammatory, immunomodulatory and drug-associated myelosuppressive effects. Considering the existing link between cancer, immune cells, inflammation and cytokines, bone marrow derived DCs based screening systems can efficiently contribute towards better understanding of the anti-cancer potential of cytotoxics. The downregulation of pro-inflammatory cytokines and chemokines and upregulation of anti-inflammatory cytokines secreted by key immune cell populations, such as DCs indicate the ability of test compounds to induce better anti-cancer effects.
Selected active compounds can further be evaluated using *in vivo* systems, which would then require lesser number of animals. Another important application of bone marrow derived cells-based assays involves *in vitro* evaluation of hematotoxic potential as an adverse side-effect induced by anti-cancer compounds using CFU assays. The conventional *in vivo* methods to study the bone marrow toxicity involve analyzing the myelosuppressive effects of the drug in various hematopoietic organs of animals. *In vitro* CFU assays can serve as alternative approach to evaluate bone marrow toxicity, prediction of inhibitory concentrations for anti-cancer agents and hence can be helpful in reduction of animals required for these studies.

In this study, we hereby propose an alternative approach for screening of new compounds with cytotoxic potential using *in vitro* bone marrow derived cell based assay systems. Bone marrow derived DC based assays are utilized to assess anticancer potential of novel compounds as reflected by downregulation of various stimulated pro-inflammatory markers (cytokines/chemokines) - the key players in cancer development. CFU-GM assays are developed from murine and human bone marrow and employed to investigate hematotoxic side effects of test anticancer compounds/formulations. Such cell based *in vitro* systems are expected to result in substantial decrease in the number of test animals, time, cost and other direct and indirect resources required. These sophisticated cell based assay systems can be employed to monitor modulations induced by the test compounds at cellular and molecular levels and hence enable us to better understand their mechanism of action. Therefore it is likely to present a cell based system to shortlist potential compounds from a large panel. The lead compound obtained from screening library of compounds can then further be analyzed in appropriate *in vivo* studies for a correlation of biological activity.
1.5 Aims and Objectives

The goal of this study was to develop \textit{in vitro} bone marrow derived cell based assays to investigate anticancer potential and hematological toxicities of chemotherapeutics.

The main objectives of the present study were:

\textbf{a)} \textbf{Assay development}

i) To develop \textit{in vitro} murine Bone marrow derived Dendritic cells (DC) assays.

ii) To develop \textit{in vitro} murine Bone marrow derived Colony Forming assays.

\textbf{b)} \textbf{Screening}

i) To screen library of new molecules for potential immunomodulatory activity using DC based assay and identify hit compounds.

ii) To determine Hematotoxic-toxicity of potential anti-cancer molecules.

\textbf{c)} \textbf{In vitro-in vivo correlation}

i) To develop \textit{in vivo} model for inflammation and study immunomodulatory activity of a selected hit.

ii) Comparison of hematological toxicity as assessed using colony forming assays with comparative toxicity performed in rodents.