CHAPTER 1

INTRODUCTION

1.1 INFLAMMATION

Inflammation, derived from the latin word *inflammatio* meaning to set on fire, is the complex biological response to harmful stimuli and irritants. It is a protective, beneficial attempt, elicited by a vast array of agents, in the host organism to remove the injurious stimuli, invading pathogens and to initiate the healing process for the tissue (Table 1.1).

Inflammatory response is divided into innate immunity and adaptive immunity. The innate immune system mounts the initial response to tissue invasion. Vasodilatation, increased vascular permeability and cellular infiltration due to sepsis, severe trauma, etc are part of the innate immune response. The primary cellular components of the innate immune system are neutrophils, macrophages, dendritic cells, and natural killer (NK) cells. In addition to these cellular components, vasodilators and cytokines also play important roles in innate immunity. The classical cytokine-secreting cells of the innate immune system are macrophages. Dendritic cells were also recently recognised as important effector cells for microbial recognition and cytokine production during innate immunity (Granucci et al 2003). The effects of cytokines are pleiotropic and redundant (Liew 2003). One cytokine has numerous functions. For example, the classic examples are TNF-α and IL-1β. Both of these cytokines have the capacity to induce fever, stimulate the production of acute-phase proteins by the liver and cause endothelial cell
activation (Dinarello 2000). Cytokines are therefore multifunctional groups of proteins that frequently have overlapping functions. For this reason, the blockade of a single cytokine will often have limited effects on the overall inflammatory response (Dinarello 1997).

**Table 1.1 Various Inflammatory stimuli**

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<th>Inflammatory Stimuli</th>
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<td>Mechanical</td>
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<td>Injury, pressure, foreign body</td>
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<td>Physical</td>
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<td>UV, Ionising radiation, temperature</td>
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<td>Chemical</td>
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<td>Acids, bases, heavy metals, bacterial toxins</td>
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<td>Allergens</td>
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<td>Immune complexes</td>
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<td>Biological</td>
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<td>Microorganisms, parasites</td>
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<td>Endogenous</td>
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<td>Tumours, enzymatic malfunctions, toxic metabolic products</td>
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An inflammatory response can also be divided into two major categories acute and chronic based on timing and pathological features. An acute inflammatory response starts within minutes and lasts for days or a few weeks, while a chronic inflammation could last for months or even years and it may arise from acute inflammation or it starts to be chronic from the very beginning. Sepsis, severe trauma and major surgery all have major acute inflammatory components. It is characterised by vasodilatation, the exudation of protein-rich fluid (plasma) and a migration of cells (primarily neutrophils) into the site of injury. Vasodilatation is a classic feature of acute inflammation. The purpose of the vasodilatory response is to facilitate the local delivery of soluble mediators and inflammatory cells.

An acute inflammatory response may be triggered by activation of tissue resident phagocytes through LPS, a cell wall component of
gram-negative bacteria (Koj 1985). This provokes the secretion of quite a number of proteins and other agents, among them the proinflammatory cytokines IL-1β, IL-6 and TNFα (Matsushima and Oppenheim 1989). Subsequently, IL-1β and TNFα lead to an activation of fibroblasts and endothelial cells and a secondary release of cytokines by these cells. The combined action of these proinflammatory agents mediates the above mentioned local changes, thus initiating focal leukocyte recruitment represented in Figure 1.1 (Moser et al 1992, Moser et al 1989, Muir 1992).

Figure 1.1  The inflammatory mechanism

Innate immunity provides an early response to inflammatory stimuli, for example to infection and regulates the onset of adaptive immunity. Adaptive immunity is mediated by specific T cells and B cells. Cytokines produced by cells of specific immunity further amplifies the inflammatory response by causing macrophage activation and stimulating the cytolytic functions (Stout and Sutlles 1997). The immune response is tightly controlled
and usually functions effectively to limit infection and promote tissue repair. A balance normally exists between proinflammatory (TNF-α, IL-1β, IL-12 and IFN-γ) and anti-inflammatory mediators (IL-10, TGF-β and certain prostaglandins). The anti-inflammatory mediators serves as an important regulator of the inflammatory response (Bai et al 1997; Oberholzer et al 2002). The ability of the immune system to mount response to a disease is dependent on many complex interactions between the components of innate and adaptive immune system.

Prolonged activation of cells of Adaptive and Innate immunity leads to chronic inflammatory diseases and autoimmune disorders (Liblau et al 1995). Chronic inflammatory diseases include rheumatoid arthritis, systemic lupus erythematosus, silicosis, atherosclerosis and inflammatory bowel disease. These disorders are characterised by a prolonged duration (weeks to months to years) in which active inflammation, tissue destruction and attempts at tissue repair are occurring simultaneously (Liew 2003). Infiltration of mononuclear cells and fibrosis are typical histological features of chronic inflammation (Davies et al 2003). Thus, disturbances in the system of interaction between immunocompetent cells lead to the inappropriate functioning or lose of their normal functioning. The state of the immune system can be in many instances the decisive factor in the development of a variety of disorders in man. It’s now suggested that the key to good health may lie in finding the right balance between the body's intricate pro-inflammatory and anti-inflammatory forces. Hence regulation of mediators of immune responses is necessary to alleviate the diseased condition.
1.2 INFLAMMATORY DISORDERS: AN ABNORMAL INFLAMMATORY RESPONSE

Inflammation, as a key defensive process in the host, is spatially and temporally tightly controlled by a regulatory mechanism. Loss of control over the regulatory mechanism can lead to a number of diseases vis-à-vis rheumatoid arthritis, chronic inflammatory bowel diseases, neurodegenerative disorders, and septic shock syndrome (Monaco et al 2004). Although the inflammatory responses are different in various diseases, they can be characterised by a common spectrum of genes and endogenous mediators involved, including growth factors, inflammatory cytokines such as interleukin1β, tumour necrosis factor-α, interleukin-6, chemokines (Macrophage Inflammatory Factor - MIP-1α/β, IL-8), matrix metalloproteinases, and toxic molecules such as nitric oxide or free radicals (Heller et al 1997, Feldmann and Maini 2003).

Rheumatoid arthritis is a chronic and systemic autoimmune disorder that is characterised by progressive destruction of synovial cartilage and bone. The etiology of rheumatoid arthritis has still not been elucidated, but it is thought to be triggered by a combination of genetic susceptibility and exposure to environmental factors. TNFα, IL-1β, IL-6, and IL-8 are significantly abundant in joint lesions of rheumatoid arthritis patients. These cytokines are mostly released from the macrophages that infiltrate joint lesions (Sweeney and Firestein 2001, Kumar et al 2001). Monoclonal antibodies against TNF-α or TNF-α receptor-Fc fusion protein suppresses the symptoms and disease progression of rheumatoid arthritis (Weinblatt et al 1999, Williams et al 1992, Feldmann and Maini 2001). Similarly, patients treated with the IL-1 receptor antagonist showed improvement in clinical studies (Garces 2001).
Inflammatory bowel disease encompasses a number of chronic, relapsing inflammatory disorders involving the gastrointestinal tract. Ulcerative colitis and Crohn’s disease are two entities of chronic inflammatory bowel diseases in which a dysregulated immune response triggered by products of the enteric bacterial flora, viral and perhaps, dietary antigens, is one of the main pathogenic mechanisms (van der Blink et al 2002). TNFα plays a central role in the initiation and amplification of the inflammatory reaction in Crohn’s disease (van Montfrans et al 2002). Monoclonal antibodies against TNF have proven clinically effective (Van Deventer 2002).

Tissue damage in acute and chronic neurodegenerative diseases is a result of a complex pathophysiological cascade which comprises a variety of distinct pathological events (Koistinaho and Koistinaho 2005). One of the common pathophysiological hallmarks of neurodegenerative disorders, such as Alzheimer’s disease, Parkinson’s disease, HIV-associated dementia, brain trauma, and stroke, is microglial activation (Danton and Dietrich 2003, Schwartz 2003). Resident non-neuronal brain cells respond rapidly to neuronal cell death and may have both deleterious and beneficial roles in neuronal damage. Microglial cells activated in vitro produce toxic reactive oxygen radicals and NO, substances that may directly damage neurons. Activated microglia release neurotoxic and proinflammatory cytokines such as FasL, TNFα, IL-1, IL-6, INFγ, and numerous chemokines (Rothwell 1999, Touzani et al 1999, Martin-Villalba et al 1999, Barone et al 1997). Cytokines produced by microglia may further activate astrocytes that in turn become a source of neurotoxic cytokines. Growing evidence indicates that the inhibition of secretion or activity of IL-1β and TNFα with neutralising antibodies or soluble cytokine receptors leads to a decrease in neuronal damage (Schielke et al 1998, Boutin et al 2001). Knockout mice deficient in neuronal nitric oxide synthase (Huang et al 1994) or IL-1β converting enzyme-ICE
(Schielke et al 1998) is less vulnerable to toxic insult. TNFα is one of the mediators, dramatically increased after brain injury that leads to the activation, proliferation, and hypertrophy of phagocytic cells and gliosis. Its inhibition by pharmacological agents, neutralizing antibodies or soluble receptors has protective effects.

Astrocytes, the major glial cell type in the brain, are important contributors to inflammatory immune responses within the brain. Astrocyte hypertrophy and proliferation (called reactive gliosis) is a widespread response to damaged neurons. Astrocytes produce several neurotrophic substances that regulate viability of neurons after ischemia, but they are also a source of pro-inflammatory (IL-1, IL-6) and cytotoxic cytokines (FasL, TNFα, TGFβ). Activated astrocytes also produce toxic molecules such as reactive oxygen species and NO (Stoll et al 1998).

1.3 CELLULAR CONSTITUENTS OF IMMUNE SYSTEM

The immune system is composed of many interdependent cell types, having specialized function, that collectively protect the body from bacterial, parasitic, fungal, viral infections and from the growth of tumor cells. These cells depend on the T helper subset for activation signals in the form of secretions formally known as cytokines, lymphokines, or more specifically interleukins.

1.3.1 Macrophages

Macrophages are bone-marrow-derived phagocytic cells that are important for tissue homeostasis and are found in virtually all tissues of the body. In the blood, they are referred to as monocytes and are a major source of pro-inflammatory cytokines. They are also often referred to as scavengers or antigen-presenting cells (APC) because they phagocytose foreign materials
and present the digested products as antigens to other cells of the immune system such as T cells and B cells. This is one of the important first steps in the initiation of an immune response. Stimulated macrophages exhibit increased levels of phagocytosis and are also secretory.

Macrophages also play an important role providing co-stimulatory ligands and co-stimulatory cytokines (e.g., IL-1β and IL-12) to the infiltrating T cells (Chambers and Allison 1997; McAdam et al 1998; Dinarello 1997; Trinchieri 1995). Macrophages can be activated to produce prodigious amounts of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6, chemoattractant cytokines such as IL-8, macrophage inflammatory proteins and pro-inflammatory products of arachidonic acid metabolism and toxic reactive oxygen and nitrogen intermediates (Dinarello 2000; Stout et al 1997; MacMicking et al 1997; Bogdan et al 2000).

Interestingly, in addition to the inflammatory and destructive mediators listed above, macrophages have the potential to contribute to the remission of the inflammatory episode, although the degree to which they participate in remission has not yet been directly assessed. Macrophages also can be induced to secrete cytokines, which inhibit macrophage accessory, inflammatory, and effector functions. IL-10, which is produced by macrophages and T cells, down-regulates expression of co-stimulatory molecules (Ding et al 1993; Buelens et al 1995), inhibits the production of IL-1β and TNF-α and reduces generation of reactive oxygen and nitrogen intermediates (Gazzinelli et al 1992; Dai et al 1996; Koch et al 1996; Oberholzer et al 2002).
1.3.2 T cells

T cells play a central regulatory role in immune and autoimmune response. T cells develop, with their specificity to recognise specific major histocompatibility (MHC)–peptide complexes on antigen-presenting cells (APCs). After the receipt of signals through the T-cell receptor (TCR) and co-stimulator, these cells are triggered to produce IL-2 and enter the cell cycle (Howland et al 2000). After several days of rapid cell division, these T cells differentiate into effector cells. During this process of differentiation, a new pattern of cytokine expression is established that provides the gene products that are responsible for the specific effector functions of these cells, and therefore their ability to protect the host from a variety of pathogens (Romagnani et al 2000, Murphy and Reiner 2002).

T cell activation plays a central role in the regulation of both normal and pathogenic condition leading to acute and chronic immune responses (Lucey et al 1996). Antigen specific CD4+ T cells direct host effector functions by means of one of two alternative cytokine responses: Th1 (cell-mediated proinflammatory responses) or Th2 (antibody-mediated responses). Th1 lymphocytes secrete proinflammatory cytokines eg. IL-2, IFN-γ, IL-12, and lymphotoxin and mediate delayed-type hypersensitivity (DTH) responses. In many cases, autoimmune diseases appear to be Th1-mediated or Th1-dependent (Karlsson et al 2000, Whitacre et al 1999, Tsiavou et al 2004). Th2 cells favour humoral-mediated responses and defend the host against extracellular parasites such as helminths and take part in atopic and allergic reactions (Romagnani et al 2000). The signature Th2 cytokine is interleukin (IL)-4 which can be measured early in Th2 development, others include IL-5, IL-6, IL-9, IL-10 and IL-13 (Glimcher and Murphy 2000, Romagnani et al 2000). Importantly, Th2 cells secrete anti-inflammatory cytokines
(e.g. IL-4, IL-5, IL-10), which are associated with down regulation of Th1 cytokines and may provide protection from Th1-mediated autoimmune disease (Cua et al 1999, Mueller et al 1996, Bettelli et al 1998).

T cell activation, which plays a central role in the regulation of both normal and pathogenic condition leading to acute and chronic immune responses, involves multiple intracellular signalling events emanating from the cell surface, TCR-CD3 complex and co-stimulatory molecules. These signalling events can be largely mimicked by stimulation of the T cells, such as combined treatment with anti-CD3 mAb and the PKC activators, PHA, PMA (Alberola-Ila et al 1997). It has been shown that under both physiological and artificial conditions of stimulation, various signal transduction pathways such as MAPK, NFAT (Tsukamoto et al 2004, Dong et al 2002) come into play. All these would ultimately integrate, leading to the secretion of cytokines and the acquisition of effector functions by the T cells.

1.4 IMMUNE SYSTEM MEDIATORS - CYTOKINES

Cytokines regulate proliferation, differentiation and maturation of various types of lymphoid cells and accessory cells of the immune system. Cytokines regulate the immune system by pleiotropy, redundancy, synergistic and antagonistic effects on various cell types. Cytokines elicit their actions through binding to specific high affinity receptor(s). Like hormones in the endocrine system, cytokines serve as messengers in the immune system. In contrast to hormone synthesis, cytokine production is not constitutive but a carefully regulated process (Kuby 1994). Cytokines trigger signal transduction and gene activation cascades that regulate cellular activation, proliferation, differentiation, and survival. Thus, the functioning of the immune system is finely balanced by the activities of pro-inflammatory and anti-inflammatory mediators or cytokines (Dinarello 2000).
1.4.1 Proinflammatory Cytokines

1.4.1.1 Tumor necrosis factor

Tumor necrosis factor alpha (TNFα), named for its ability to cause rapid necrotic tumor regression, is the founding member of the TNF ligand superfamily, which is known to have pleiotropic functions including cell proliferation, differentiation, activation, and apoptosis (Locksley et al 2001). TNFα is primarily produced by macrophages, lymphocytes, neutrophils, endothelial cells, keratinocytes and fibroblasts during acute inflammatory reactions (Baud and Karin 2001, Medzhitov 2001). Early expression of cytokines and chemokines is important to upregulate adhesion molecules to bring other responding cells to the site (Tak et al 1996). TNF-α expression also leads to activation of macrophages, which improves phagocytosis and antigen presentation. Pro-inflammatory cytokines, which are over-expressed in RA joints (Feldmann et al 1992, Firestein et al 1990), have been shown to induce a variety of disease genes, including other cytokines, proteases, oxygenases, and adhesion molecules, as well as to stimulate cell proliferation (Feldmann et al 1996, Koch et al 1995).

Among these factors, TNFα has received the greatest attention because of its position at the apex of the pro-inflammatory cytokine cascade, and its dominance in the pathogenesis of RA (Feldmann and Maini 2001). Many lines of evidence support this theory. (1) TNFα is expressed at high levels in inflamed synovium, particularly at the cartilage-pannus junction, from RA patients (Di Giovine et al 1988, Firestein et al 1990, Saxne et al 1988), (2) anti-TNFα inhibits the production of other pro-inflammatory cytokines including IL-1, IL-6, IL-8 and GM-CSF (Brennan et al 1989), and (3) by itself, TNFα can induce joint inflammation and proliferation of fibroblast-like synoviocytes (FLS) (Gitter et al 1989), trigger cartilage destruction by inducing collagenase (Dayer et al 1985, Dayer et al 1986),
inhibit proteoglycan synthesis by articular chondrocytes (Saklatvala 1986, Saklatvala et al 1985), and stimulate osteoclastogenesis and bone resorption (Abu-Amer et al 2000, Bertolini et al 1986). Early TNF-α expression is important for innate responses to infection and to improve interactions between macrophages and T cells. Later TNF-α is produced by many cells including T-cells, macrophages and NK cells (Tracey and Cerami 1994). TNF-α stimulates the production of many inflammatory cytokines such as IL-1β, IFN-γ, NO, iNOS etc (Cheshire and Baldwin 1997; Tracey and Cerami 1994; Dinarello 1997). Over expression of this cytokine plays an important role in chronic inflammatory diseases such as multiple sclerosis or rheumatoid arthritis (Lipsky et al 2000; Feldmann 1996; Rutgeerts et al 1999; Lamprecht et al 2002; Aeberli et al 2002; Sfikakis 2002). Thus TNF-α is well known for its proinflammatory effects and its inhibition has unquestionably been an enormously beneficial strategy. Based on these studies there are now two anti-TNF treatments, etanercept (a soluble TNFR2-IgG1 fusion protein) and infliximab (a chimeric monoclonal antibody against TNFα), that have been approved by the Food and Drug Administration and the European Medicine Evaluation Agency for RA (Choy and Panayi 2001).

1.4.1.2 IL-1 Beta

IL-1β is one of the prototypic multifunctional cytokines, which can affect almost all types of cells and induce its effect frequently with other cytokines and mediator molecules. IL-1β was first discovered as a major mediator of inflammation, it has gradually become evident that this cytokine has numerous functions related to host defence mechanisms (O’Neill and Dinarello 2000; Dinarello 1997), regulating not only the immune system, but also the areas of the neuronal and endocrine systems that interface with the immune system (Tocci et al 1997). IL-1 is produced by various types of cells, including macrophages, dendritic cells (O’Neill and Dinarello 2000;
Dinarello 1997), B cells, and T cells. In the immune system, IL-1β is known to activate lymphocytes, monocytes, macrophages, and NK cells (Dinarello 2000; Dinarello 1997).

IL-1 has diverse potentiating effects on the proliferation, differentiation, and functioning of diverse non-adaptive cells (NK cells, macrophages, granulocytes, etc.) as well as specific immunocompetent cells (T and B cells). Initially, IL-1 was characterised as ‘the classical’ co-stimulatory cytokine for T cell proliferation, inducing IL-2 secretion and expression of IL-2Rs by activated T cells (Yoza et al 1992; Brooks et al 1995; Dinarello 2000). IL-1 is also required for maximal-IL-12 driven TH1 development, possibly through activation of NK cells for IFN-γ production (Shibuya et al 1998).

For IL-1, the most salient and relevant properties are the initiation of cyclooxygenase type 2 (COX-2), type 2 phospholipase A and inducible nitric oxide synthase (iNOS). This accounts for the large amount of prostaglandin-E2 (PGE2), platelet activating factor and nitric oxide (NO) produced by cells exposed to IL-1 or in animals or humans injected with IL-1. This latter property promotes the infiltration of inflammatory and immunocompetent cells into the extravascular space. Thus, IL-1 is the primarily proinflammatory cytokine by their ability to stimulate the expression of other inflammatory genes (Takabayashi et al 2004; Dinarello 2002) and is associated with inflammation and autoimmune diseases (Bolon et al 2004; Dayer 2004; Thomas and Chhabra 2003). Regulation or inhibition of interferon-beta has been shown to have therapeutic value (Dinarello 1997).

1.4.1.3 IL-8 - The Proinflammatory Chemokine

IL-8 was first purified as a chemotactic factor for neutrophils (Yoshimura et al 1987). However, subsequent studies demonstrate that IL-8

IL-8 induces T cell chemotaxis in vitro and several lines of evidence suggest potential roles of IL-8 in vivo T cell migration (Larsen et al 1989; Kudo et al 1991). A subcutaneous injection of IL-8 caused T lymphocyte migration into the injected site after neutrophils migrated (Detmers et al 1990). Moreover depletion of neutrophils diminished IL-8 induced CD4+ T lymphocyte migration in the later phase (Kudo et al 1991) suggesting that IL-8 induced CD4+ T lymphocyte migration indirectly by chemo-attracting neutrophils. IL-8 upregulated Th1 derived cytokines IFN-γ, TNF-α and IL-2 in vitro. In contrast Th2 derived cytokines IL4, IL10 and IL13 inhibited the chemotaxis of T lymphocytes to IL-8 (Jinquan et al 1995). These results suggest the participation of IL-8 in Th1 mediated immune reactions. This notion may be supported by the observation that treatment with an anti IL-8 antibody reduced lymphocyte as well as neutrophil infiltration in a Th1 mediated delayed type hypersensitivity tuberculin reaction (Larsen et al 1995). Thus IL-8 is a known putative marker of several inflammatory and autoimmune diseases (Seitz et al 1992; Hayashida et al 2001; Goldenberg-Cohen et al 2004; An et al 2004).
1.5 PROINFLAMMATORY MEDIATORS IN INFLAMMATION

1.5.1 Nitric Oxide

Since its discovery, nitric oxide (NO) has become one of the most highly studied and important biological molecules. There are now known to be several NO synthases including nNOS (neuronal NOS), eNOS (endothelial NOS), and iNOS (inducible NOS). The three forms display approximately 50% identity in amino acid sequence. Both nNOS (150 kDa) and eNOS (135 kDa) are constitutive enzymes. Activation of eNOS and nNOS are rapid and however, the output of these enzymes is transient and low, on the nanomolar level. NO at this level has a relatively long half-life and is mainly involved in homeostatic processes such as neurotransmission, peristalsis, and blood pressure regulation.

In contrast to the constitutive enzymes, iNOS is the inducible form of the enzyme (130 kDa). In stimulated macrophages, iNOS may produce NO at $10^6$ molecules/cells (Lewis et al. 1995). In cases where inflammation continues over months or even years, neighbouring cells may be exposed to significant quantities of highly reactive chemical species including NO and reactive oxygen species. It is now understood that many types of cells can be activated to express iNOS in the presence of the appropriate mix of cytokines.

A particularly important role of NO is its involvement in host defence (Hibbs 2002). NO is produced by macrophages as a cytotoxic agent in the immune or inflammatory response (MacMicking et al. 1997; Moncada et al. 1991). NO is released in combination with numerous other species including hydrogen peroxide, FAS ligand, tumor necrosis factor, and inflammatory cytokines. However, NO is believed to be the key mediator of macrophage-induced cytostasis because NO scavengers block the cytostatic effect of macrophages (Moncada et al. 1991; Hibbs 2002). In regions of
inflammation, particularly chronic inflammation, local NO production can be quite high resulting in larger amounts available for further reaction with oxygen or superoxide to generate additional highly reactive species (Bogdan et al 2000). It has been proposed that reactive oxygen species formed during the inflammatory response may play a role in DNA damage and cellular injury (Murphy 1999; Amin and Abramson 1998).

iNOS levels in activated macrophages are regulated by a variety of anti-inflammatory cytokines. Studies from mice lacking IL-10 or TGF-β1 have shown that both the cytokines play essential role in negative regulation of iNOS (Murray and Young 1999). The third class includes IL-4 and IL-13, related cytokines that are powerful inhibitors of NO production from activated macrophages (Bogdan et al 1994; Doyle et al 1994).

1.5.2 Mast Cells

Mast cells, which are constituents of virtually all organs and tissue, are important mediators of inflammatory responses such as allergic inflammation and hypersensitivity. In response to the antigen cross-linking of immunoglobulin E (IgE) receptor or direct activation, mast cells start releasing mediators, such as histamine, inflammatory cytokines, and products of arachidonic acid metabolism (Beaven and Metzger 1993, Church and Levi-Schaffer 1997, Metcalfe et al 1981). Several inflammatory and chemotactic cytokines, such as tumor necrosis factor (TNFα), interleukin (IL)-6, IL-8, IL-4, IL-13 and the transforming growth factor β are produced from activated mast cells (Bradding et al 1993, Burd et al 1989, Plaut et al 1989). Pro-inflammatory cytokines, including TNFα and IL-6 support the well-recognised role of mast cells in allergic inflammation and hypersensitivity. TNFα induces tissue damage and is considered a major
initiator of inflammation. IL-6 is a pleiotropic inflammatory cytokine produced by mast cells (Mican et al. 1992, Walsh et al. 1991).

1.5.3 Arachidonic Acid Pathway Enzymes - Phospholipase-A₂ and Cyclooxygenase-2

Cytosolic phospholipase A₂ alpha is a ubiquitous, intracellular mammalian enzyme that catalyzes the release of arachidonic acid from membrane phospholipid (Hirabayashi and Shimizu 2000, Leslie 1997). Arachidonic acid is the precursor for a variety of potent biologically active metabolites including the prostaglandins and leukotrienes (Figure 1.2). The eicosanoids are important mediators of acute inflammation but also play diverse roles in regulating normal physiological processes such as homeostasis, parturition, and kidney function (Funk 2001). The conversion of arachidonic acid to prostaglandins occurs through the cyclooxygenase (COX) pathway by the constitutive enzyme, COX-1, or by the inducible COX-2 (Smith and Langenbach 2001). Arachidonic acid is also converted to leukotrienes involved in inflammation including the cysteinyl leukotrienes (LTC₄, LTD₄, LTE₄) and leukotriene B₄ by 5-lipoxygenase (5-LOX). The amount of arachidonic acid released from membrane phospholipids is tightly regulated and is important for controlling the production of eicosanoids.

As the first enzyme in the cascade, cPLA₂ is subject to complex mechanisms of activation, which occurs primarily by posttranslational processes. PLA₂ is regulated by calcium and phosphorylation, which act together for full activation (Hirabayashi and Shimizu 2000, Leslie 1997). Certain agonists activate cPLA₂ by increasing the intracellular calcium concentration ([Ca²⁺]i) (Lin et al. 1992), allowing cPLA₂ to bind calcium through the C2 domain and translocate from the cytosol to Golgi,
endoplasmic reticulum (ER) and the nuclear envelope (Evans et al. 2001, Glover et al. 1995, Hirabayashi et al. 1999, Perisic et al. 1999, Schievella et al. 1995). The arachidonic acid released from these sites couples to cyclooxygenases, which are predominantly localized to the luminal surface of ER and nuclear envelope (Spencer et al. 1998). The nucleus is also an important site of leukotriene synthesis (Peters-Golden and Brock 2001).

Cyclooxygenase (COX) catalyses the two-step conversion of arachidonic acid to prostaglandin H$_2$, the first reaction required for the biosynthesis of various types of prostaglandins and thromboxanes, which are

Figure 1.2 Role of arachidonic acid metabolism in Inflammation
implicated in various physiological events including progression of inflammation, immunomodulation, and transmission of pain (Vane et al 1998). COX-2 expression is proved to be increased at the sites of inflammation and COX-2 expression is reported in airway cells from patients with asthma and chronic obstructive pulmonary disease (Redington et al 2001). Previously, studies also reported that COX-2 could be induced, after T cell receptor triggering, having functional implications in T cell function (Iniguez et al 2000, Iniguez et al 1999). Moreover, a recent result indicates that in vivo suppression of COX-2 by glucocorticoids, in T cells and not in macrophages is required to prevent the deleterious role of this enzyme in inflammation (Brewer et al 2003). Therefore, current research on anti-inflammatory drugs has been aimed at targeting the COX-2 inducible production of prostaglandins (Dannhardt and Ulbrich 2001).

1.6 TH1 / TH2 HYPOTHESIS IN IMMUNE REGULATION

One theory of immune regulation involves homeostasis between T-helper 1 (Th1) and T-helper 2 (Th2) activity. Differentiation of naive, Th precursor cells into Th1 or Th2 cells is a complex developmental process, and molecular mechanisms underlying that may provide a conceptual framework in developing immune modulation therapies against allograft rejection, autoimmune diseases, and allergic diseases. Th1 cells produce cytokines that are involved in the attack against intracellular pathogens such as viruses, raise the classic delayed-type hypersensitivity (DTH) skin response to viral and bacterial antigens, and fight cancer cells. Th2 cells play a role in protection against extracellular pathogens such as multicellular parasites. (Figure 1.3)
Figure 1.3 Regulation of Th1 and Th2 responses

IL-12 and IFN-γ are the predominant cytokines that control the differentiation program of Th0 into Th1 cells. On the negative side, the Th1 pathway when it over reactive, can generate organ-specific autoimmune disease including rheumatoid arthritis, insulin-dependent diabetes mellitus, experimental autoimmune encephalitis, and others (Schulze-Koops et al 2001). Similarly the dominant factors that control the differentiation program of Th0 into Th2 is IL-4. Th2 cells produce IL-4, IL-5, IL-10, which evoke strong antibody responses, eosinophil accumulation and promote allergic responses (Lee et al 2000; Romagnani 2000), but inhibit several functions of phagocytic
cells and Th1 cells. Thus, the two systems are inter-related, either pathway can down-regulate the other and over activation of either pattern can cause diseases.

1.6.1 IL-2

IL-2 is a multifunctional cytokine, which principally functions as an inevitable cytokine in stimulating both humoral and cell-mediated immunity. Basically, IL-2 is an essential growth factor for T cell cycle progression and proliferation (Smith 1988). IL-2 is important not only for T cell survival but also for proliferation and differentiation of T cell responses into Th1 or Th2 predominance (Mosmann et al 1996). Inadequate IL-2 synthesis may lead to T cell death or induction of clonal anergy (Schwartz 1990; Go and Miller 1992). Furthermore, IL-2 has been known to bring about an array of biological effects on other cell types besides T cells including Natural killer cells, B cells, Monocytes, Neutrophils and Eosinophils (Smith 1988). The IL-2 has a role in the destruction of target cell lysis by its ability to stimulate NK cell and CD8+ T cells.

In addition, IL-2 can synergise with other cytokines such as IL-15 and act as chemoattractants for T cells. IL-2 stimulates the proliferation of NK cells, facilitate their synthesis of IFN-γ and TNF-α (Smith et al 1988) and induce the proliferation and immunoglobulin synthesis by human B cells stimulated with anti-IgM. Studies also show that IL-2 contributes to the increased production of proinflammatory cytokines. IL-2 enhances allergic airway responses in rats by increased inflammation (Renzi et al 1999). The physiological stimulating signals for expression of the IL-2 gene which is transiently induced by the combinational immune stimuli is provided by the interaction of T-cell antigen receptor complex with antigen MHC complex together with accessory signals from the antigen-presenting cell. This can be mimicked by T-cell specific mitogens including phytohemagglutinin and Con
A or by the simultaneous presence of phorbol esters and calcium ionophores (Truneh et al 1985; Kumagai et al 1987). IL-2 gene expression is tightly regulated at the transcriptional level with no basal level of expression (Go and Miller 1992; Hughes and Pober 1996, Weiss and Littman 1994, Whitehurst and Geppert 1996). Drugs affecting IL-2-dependent signaling have been proved as extremely useful and potent immunosuppressive drugs in organ transplantation and are also employed to treat some autoimmune diseases (Sigal and Dumont 1992; Thomson and Starzl 1993; Kugathasan et al 1998; Renzi et al 1999).

1.6.2 Biological importance of Th1 cytokines-its role in immunological disorders

1.6.2.1 Interleukin-12

Interleukin-12, a heterodimeric cytokine plays a central role in the immune response as a cytokine whose functions bridge innate resistance and Ag specific adaptive immunity (Trinchieri 1995). It is produced by phagocytic cells and APCs, in particular macrophages and dendritic cells as the result of interaction with bacteria, bacterial products such as LPS, and intracellular parasites (Macatonia et al 1995). IL-12 has attracted attention because of its potential in driving the differentiation of naive CD4+ T (Macatonia et al 1995) and in reversing the polarised Th2 cells to Th0 and Th1 phenotype (Smits et al 2001). IL-12 stimulates IFN-γ production by NK and T cells and induces the development and differentiation of Th1 cells (Trinchieri 1995; Barbulescu et al 1998). IL-12 upregulates a variety of co-stimulatory molecules and MHC class II and downregulation by IL-4 and IL-10 (Koch et al 1996). Since IL-12 plays critical role in driving Th1 T cell responses, overproduction of IL-12 can be dangerous to the host, e.g., in the immunopathology of organ-specific autoimmune diseases.
(Simpson et al. 1998; Gately et al. 1998, Nicoletti et al. 1996; McDyer et al. 1998), suggesting that the level of this cytokine must be tightly controlled (Neurath et al. 1995).

1.6.2.2 Interferon-gamma (IFN-γ)

Interferon-gamma (IFN-γ) is a cytokine that plays physiologically important roles in promoting innate and adaptive immune responses (Farrar and Schreiber 1993; Rogge et al. 1998; Cheshire and Baldwin 1997). IFN-γ is a member of a family of proteins originally identified by their capacity to non-specifically protect cells from viral infection. These proteins have since been divided into two classes on the basis of structural and functional criteria as well as the stimuli that induce their expression. Type I IFNs are primarily induced in response to viral infection of cells and have been further subdivided into two groups (IFN-α and IFN-β) based on their cellular origin. Type II or immune IFN, now known as IFN-γ, is produced predominantly by T lymphocytes, NKT cells and natural killer (NK) cells following activation with immune and inflammatory stimuli rather than viral infection. One of the major physiologic roles of IFN-γ is its ability to regulate MHC class I and class II protein expression on a variety of immunologically important cell types (macrophages, monocytes, endothelial and epithelial cells). At the functional level, IFN-γ dependent up-regulation of MHC gene expression is an important step in promoting antigen presentation during the inductive phase of the immune response (Farrar and Schreiber 1993).

Mice deficient in IFN-γ or IFN-γ receptors have impaired production of macrophage antimicrobial products (like NO) and reduced expression of MHC-II antigens. Concerning an anti-infectious activity, this antibacterial effect might well be one of the main specific activities of IFN-γ
(Martin et al. 1994). Although monocyte/macrophages are a prime cellular target for IFN-\(\gamma\) under physiological conditions, IFN-\(\gamma\) exerts its effects on other cells of the immune system. It regulates IgG switching in B-cells and antagonises the ability of IL-4 to induce MHC class II expression on murine B-cells. IFN-\(\gamma\) upregulates a variety of pro-inflammatory parameters such as interleukin (IL)-12, tumor necrosis factor-a (TNF-\(\alpha\)), interferon-inducible protein-1), inducible nitric oxide synthase (iNOS). Moreover, data suggest that IFNg may also be able to enhance activation of the pro-inflammatory transcription factor nuclear factor-kB (NF-kB) under certain conditions (Cheshire and Baldwin 1997). In accord with these functional characteristics, bioactivity of IFN-\(\gamma\) has been identified as a prerequisite in several models of inflammatory and autoimmune diseases, e.g., insulin-dependent diabetes mellitus, myas-thenia gravis (Zhang et al. 1999), thyroiditis (Alimi et al. 1998), as well as lupus nephritis (Schwarting et al. 1998) and crescentic glomerulonephritis (Kitching et al. 1999).

On the other hand, IFN-\(\gamma\) blocks development of the Th2 subset through two mechanisms. It inhibits synthesis of IL-4 from undifferentiated antigen-stimulated T cells, thereby inhibiting production of the cytokine that is required for Th2 development (Farrar and Schreiber 1993). It also inhibits Th2 cell expansion by directly inhibiting proliferation of Th2 cells. The anti-proliferative effects of IFN-\(\gamma\) are not observed on Th1 cells because these cells do not express the IFN-\(\gamma\) R2 subunit.

1.6.3 Biological importance of Th2 cytokines-its role in immunological disorders

1.6.3.1 Interleukin -4

Interleukin-4 is a pleiotropic cytokine dictating the nature of the immune response to infectious antigens (Ags) and self-Ags through its effect
on a variety of cell populations. IL-4 plays an essential role in T and B cell differentiation where it is critical for initiating type 2 immune responses (Nelms et al 1999).

Especially during allergic activation basophils, T cells and mast cells induce local IL-4 production, which promotes responses in macrophages (Nicod et al 1997; Nicod et al 2000), class switching of B-cell to IgE production and mucus hypersecretion, eosinophil activation and recruitment. IL-4 has also been implicated in smooth muscle contraction. IL-4 mediates this function by binding to its heterodimeric receptor IL-4R. Binding of IL-4 to its receptor leads to Janus kinase (JAK) 1 and JAK3 activation. One or both of these tyrosine kinases, phosphorylate the IL-4a chain residue that is responsible for IRS-2 recruitment (Nelms et al 1999) and mobilisation of signal transducer and activator of transcription (STAT) 6 (Ryan et al 1996; Reichel et al 1997). Importantly, STAT6 is the only STAT consistently shown to associate with the IL-4 receptor (Nelms et al 1999; Takeda et al 1996; Wang et al 2000).

In addition, IL-4 is recognised as an anti-inflammatory cytokine that modulates macrophage activity through the suppression of proinflammatory cytokine expression. Macrophage-induced cytokines are important for differentiation of Th1-type cells and cytokines such as IL-4 may be essential for the regulation of Th1-type responses (Mueller et al 1996). The activity of Th1-type cells and production of IL-12, TNF-α, and IFN-γ can be directly inhibited by IL-4 (Scumpia et al 2004; Gautam et al 1992; Koch et al 1996; Wang et al 1995). The ability of Th2-associated cytokines to inhibit the development of deleterious Th1-mediated responses suggested that they might be used as therapeutics in autoimmune disease (Finnegan et al 1999; Horsfall et al 1997; Mueller et al 1996).
1.6.3.2 Interleukin -10

Interleukin-10 (IL-10) is a multifunctional cytokine produced by B cells, stimulated macrophages, and subsets of CD4+ T cells. It was originally termed cytokine synthesis inhibition factor due to its ability to inhibit production of some cytokines such as interferon-γ and interleukin-2 (IL-2) (Fiorentino et al. 1991). It has been shown to have both immunostimulatory and immunosuppressive properties, depending on the target cell type. IL-10 exerts immunostimulatory properties on B cells and mast cells and is a growth factor for immature thymocytes (Mosmann 1994). It exerts immunosuppressive effects on cells of the monocyte/macrophage lineage (Mosmann 1994) and also on subsets of activated helper CD4+ T cells (Mosmann 1994). The IL-10 receptor has been found on macrophages and B cells as well as T cells, including murine CD4+ T-cell clones. IL-10 inhibits the production of a wide range of inflammatory cytokines and NF-kB activation in both monocytes and alveolar macrophages (Thomassen et al. 1996; Armstrong et al. 1996; Wang et al. 1995). IL-10 inhibits IFN-γ secretion from activated Th1 lymphocytes and NK cells and aids in treatment of Th1 mediated disorders (Bai et al. 1997).

Several in vitro and in vivo studies have shown that IL-10 suppresses production of cytokines (IL-1β, IL-6, TNF-α, GM-CSF, IL-12), generation of reactive oxygen intermediates, and expression of surface MHC class II and co-stimulatory molecules such as B7 (Armstrong et al. 1996; Gazzinelli et al. 1992; Ding et al. 1993; Bessis et al. 1995). The deficiency in IL-10 production leads to increase in inflammatory response (Gudmundsson et al. 1998; Bonfield et al. 1995; Borish et al. 1996). Hence, induced production of IL-10 is of particular interest because of its antagonistic effects on IFN-γ, a potent activator of macrophages.
1.7 INTRACELLULAR TARGETS

1.7.1 Mitogen Activated Protein Kinases - Involvement in Regulation of Crucial Inflammatory Mediators

The mitogen-activated protein kinase (MAPK) superfamily consists of three main protein kinase families: the extracellular signal-regulated protein kinases (ERKs), the c-Jun N-terminal kinases or stress-activated protein kinases (JNK/SAPK) and the p38 family of kinases (Kyriakis and Avruch 2001; Rao 2001). Each is proving to have major roles in the regulation of intracellular metabolism and gene expression and integral actions in many areas including growth and development, disease, apoptosis and cellular responses to external stresses. Each of the MAPK modules operates as a three-tier system. The MAPK, a serine/threonine kinase, is activated by a MAPK kinase (MAPKK), which is a ‘dual-specific’ kinase that phosphorylates at both Ser/Thr and Tyr sites, targeting a Thr-X-Tyr motif on the MAPK (where X is glutamate, proline or glycine for the ERK, JNK and p38 modules, respectively) (Kyriakis and Avruch 1996).

Phosphorylation of the MAPK results in a conformational change and a >1000-fold increase in specific activity so that, in effect, MAPKs are inactive unless phosphorylated by their respective upstream kinases (Kyriakis and Avruch 2001). In turn, the MAPKK is activated by a MAPKK kinase (MAPKKK) that receives signals from stimulus-activated receptors on the cell surface or through interactions with GTP-binding proteins and/or other kinases. The MAPKs at the end of these signalling cascades phosphorylate their target proteins, many of which are nuclear proteins such as transcription factors. Hence, MAPKs have a key role in the regulation of many genes.
All MAPK cascades cooperate in the orchestration of inflammatory responses, and extensive cross-talk to other inflammatory pathways, such as NF-kB and Janus kinase/STAT signalling, has been described (Tibbles and Woodget 1999; Kyriakis and Avruch 2001).

1.7.1.1 Extracellular signal-regulated protein kinases

The pathways by which the ERKs may become activated have been studied in a number of cell systems. These kinases are known to be directly activated by mitogen-activated protein kinase, a kinase directly upstream of the ERKs. The mechanism by which MEK (MAP kinase of ERK Kinase) becomes activated varies, depending on the stimulus and the cell type. Upon macrophage or T cell activation, p21ras becomes activated and recruits raf-1 to the plasma membrane (Rao 2001, Downward et al 1990, Izquierdo et al 1993, Stokoe et al 1994, Zhang et al 1993). Raf-1 and B-Raf activates the dual specificity mitogen-activated protein (MAP) kinase kinases (MEK1 and MEK2) (Kyriakis et al 1992, Tsukamoto et al 2004), which are responsible for the subsequent activation of the MAP kinases, ERK1 and ERK2, by phosphorylation of Tyr and Thr residues (Cobb and Goldsmith 1995, Whitehurst and Geppert 1996). The activated ERKs translocate to the nucleus to target the ternary complex factor Elk-1, which controls transcription of the c-fos protooncogene (Gille et al 1995, Whitmarsh and Davis 1996).

Generally, activation of an ERK signaling pathway has a role in mediating cell division, survival, migration and production of various cytokines (Rao et al 2002). The ERK module responds primarily to growth factors and mitogens. In vitro this pathway is stimulated by various stimuli such as PHA, PMA, LPS etc (Arditi et al 1995). Mechanistic studies of this pathway were significantly augmented by the discovery of PD98059 and U0126, ERK pathway inhibitors, that bind to inactivated MEK1 and prevents its phosphorylation by Raf (Dudley et al 1995, Favata et al 1998). Use of
these soluble inhibitors has implicated MEK-ERK signaling in a variety of human and murine macrophage and T cell effector functions, including TNFα, IL-1β and IL-6 synthesis (van der Bruggen et al 1999, Means et al 2000, Carter et al 1999), protection from Fas-mediated apoptosis (Holmstrom et al 1999), inducible NO synthesis (Ajizian et al 1999) and IL-2 gene transcription in T cells (Whitehurst and Geppert 1996, Koike et al 2003). Thus, it appears that MAP kinase function, in particular Raf-MEK-ERK signaling, plays a critical role in a variety of inflammatory processes.

1.7.1.2 c-Jun N-terminal kinases

JNKs are widely expressed in many tissues. JNKs respond to a variety of stress signals including heat shock, osmotic stress, pro-inflammatory cytokines, ischemia and UV exposure (Pombo et al 1994, Irving and Bamford 2002). The JNKs are activated by dual phosphorylation at the Thr-Pro-Tyr motif by JNKK1 and JNKK2, also known as MAPK kinase 4 and 7 (MKK4 and MKK7) (Tournier et al 1999). Upstream of the MKKs are their MAPKKKs, which include MEKKs 1-4, ASK, and a member of the mixed-lineage kinases (MLKs) (Hirai et al 1996). In turn, these are activated by GTP-binding proteins of the Rho family (Teramoto et al 1996).

JNKs are active as dimers to translocate across the nuclear membrane. JNKs were originally identified as the major kinases responsible for the phosphorylation of c-jun, leading to increased activity of the AP-1 (activator protein-1) which is an important transcription factor for IL-2 synthesis (summarised in Shaulian and Karin 2002), other nuclear transcription factors are also now known to be targets including ATF-2, Elk-1, Myc, Smad3, tumor suppressor p53, NFAT4, DPC4 and MADD, a cell death domain protein (Atfi et al 1997, Zhang et al 1998). JNK-regulated transcription factors help to regulate gene expression in response to a variety
of cellular stimuli, including stress events, growth factors and cytokines (Whitmarsh et al 1995). During inflammation, activation of the JNK signaling cascade generally results in production of inflammatory mediators (Li et al 2004). IL-1β is a potent inducer of JNK phosphorylation and collagenase gene expression in RA synoviocytes (Han et al 2001). Recently, using a novel selective JNK inhibitor, studies showed that c-Jun N-terminal kinase is required for metalloproteinase expression and joint destruction in inflammatory arthritis. JNK inhibitors have also shown promise in animal models for the treatment of rheumatoid arthritis (Han et al 2001, Vincenti and Brinckerhoff 2001). Thus JNK participates in various intracellular signaling cascades resulting in inflammatory responses.

1.7.1.3 p38 MAPK kinases

The p38 MAPK has been implicated as an important regulator of the coordinated release of cytokines by immunocompetent cells and the functional response of neutrophils to inflammatory stimuli (Herlaar and Brown 1999). Many different stimuli can activate p38 MAPK. These include LPS and other bacterial products, cytokines such as TNFα and IL-1β, growth factors, and stresses such as heat shock, hypoxia, and ischemia/reperfusion (Herlaar and Brown 1999). Upstream activators of the p38 MAP kinase include MKK3, MKK4, and MKK6 (Derijard et al 1994, Raingeaud et al 1996), which phosphorylate p38 MAP kinase on Thr and Tyr.

p38 MAPK positively regulates a variety of genes involved in inflammation, such as TNF-α, IL-1, IL-6, IL-8, cyclooxygenase-2, collagenase-1 and -3. Because of the broad pro-inflammatory role of p38 MAPK in several in vitro systems, inhibition of this pathway has been advocated as a novel therapeutic strategy for inflammatory diseases (Lee et al 2000). Inhibition of p38 Mitogen-Activated Protein Kinase has
been shown to reduce the production of TNFα, IL-1β and NO production (Badger et al 1998, Underwood et al 2000, Badger et al 2000, Nick et al 2000).

1.7.2 Transcription Factors and Inflammatory Disease

Inflammation is mediated by a vast array of soluble factors, cell surface molecules and enzymes that recruit cells to the site of inflammation and activate their gene expression machinery to produce more inflammatory mediators. Transcription factors are central to this process. After a signal has been transduced from the cell surface to the nucleus, the transcription factors respond in combinatorial fashion to bind the promoter regions of genes containing appropriate recognition sequences to induce their expression at the level of mRNA synthesis. Because the genes for many inflammatory mediators contain similar recognition sequences, or response elements, it is likely that inhibition of a limited number of key transcription factors may simultaneously control many mediators of inflammation. This may overcome some of the redundancy that is inherent to biological systems, a potentially key factor responsible for the failure of therapies that target only one pathway or receptor. Transcription factors that are necessary for the expression of a large number of inflammatory mediators include NF-κB, AP-1, NFAT and STATs.

1.7.2.1 NF-κB

The transcription factor NF-κB stands out as one of the most important inducers of inflammation. It is involved in the expression of numerous inflammatory mediators, including the cytokines TNFα, IL-1β, IL-2, IL-6 and IL-8, enzymes (iNOS, COX-2, cPLA₂) and cell adhesion molecules (ICAM-1, VCAM-1 and E-Selectin) (Baldwin 2001). Not
surprisingly, then, NF-κB has a central role in a wide variety of inflammatory diseases, including RA, inflammatory bowel diseases and asthma (Barnes and Karin 1997).

In the dormant state, NF-κB resides in a cytoplasmic complex with its inhibitory proteins, IκBα and IκBβ. Activation of NF-κB occurs rapidly in many cell types in response to cytokines, growth factors, stress inducers and other stimuli. The consequent intracellular signal transduction pathways lead to activation of IκB kinases. The subsequent chain of events involves phosphorylation that leads to degradation of IκB proteins and release of NF-κB, thus allowing its translocation into the nucleus where it is available to bind recognition sequences in the promoter regions of numerous genes.

The classic components of NF-κB, RelA (p65) and p50, are found predominantly in the nuclei of synovial macrophages, both in the synovial lining and interior to the lining, as well as in a number of other types of cells, including some fibroblast-like and endothelial cells. It should be noted that the presence of nuclear NF-κB indicates that activation and nuclear translocation from the cytoplasm has taken place. Immunohistochemical evidence of expression of TNFα and IL-1β in rheumatoid synovial macrophages is consistent with the presence of activated NF-κB in these cells.

1.7.2.2 AP-1

AP-1 is a dimer that consists of proteins from the Fos and Jun family, and its activity is regulated both at the level of gene transcription and by posttranslational modifications of its components (Karin 1995, Chinenov and Kerppola 2001, Shaulian and Karin 2002). AP-1 is composed of either Fos-Jun heterodimers or Jun-Jun homodimers. Its composition may be important for cytokine gene transcription. AP-1 is involved in induction of a
variety of inflammatory mediators such as TNFα, IL-1β, chemokines etc. MAPK pathways directly regulate AP-1 dependent transcription, both at the level of de novo synthesis of AP-1 proteins, jun and fos and by controlling their transcriptional function (Karin 1995).

Once active, the MAPK travel to the nucleus where they phosphorylate and activate specific transcription factors involved in inflammatory and growth responses. All the three main subgroups of MAPK have been described in AP-1 regulation. The extracellular signal-regulated kinases (ERK), stress-activated protein kinases/c-Jun N-terminal kinases (SAPK/JNK) and p38 become active in response to cytokines, phorbol esters, growth factors, IL-1, TNFα, osmotic stress and U.V. and activate the AP-1 family members c-fos, c-jun and ATF2, which mediates transcription of the c-Jun gene (Karin 1995, Gille et al 1992, Minden et al 1994, Whitmarsh et al 1995). This suggests that all of the MAPK cascades can contribute to AP-1-dependent transcription.

1.8 SIGNAL TRANSDUCERS AND ACTIVATORS OF TRANSCRIPTION

The STATs (Signal Transducers and Activators of Transcription) have been shown to have a prominent role in immune responses. The JAK/STAT signaling pathway is broadly used by interferons and Type 1 cytokines. These cytokines and interferons activate JAKs, which, in turn, phosphorylate and thereby activate STAT proteins, the cytosolic substrate of these kinases. However, after activation, STAT proteins are translocated to the nucleus where they function as transcription factors. A published study demonstrating activation of STAT-3, or possibly a closely related STAT family member, in peripheral blood mononuclear cells, in vitro, in response to
IL-6 activity in RA synovial fluid can be considered as a model of the interaction between synovial fluid and synovial lining macrophages.

STATs are rapidly tyrosine-phosphorylated after stimulation with cytokines, and they subsequently dimerise and translocate to the nucleus, where they can activate transcription. Tyrosine phosphorylation of STATs is essential for activation, dimerisation, and DNA binding. To date, 6 distinct but homologous members of the mammalian STAT family have been identified, and designated STAT-1 through STAT-6 (Leonard and O’Shea 1998). Most STATs are widely expressed in a variety of cell types. An individual STAT protein may be activated by multiple ligands, but certain cytokines preferentially activate particular STATs. Experiments with mutant cell lines and knockout mice show that STATs are critically important for mediating many, but not all, cytokine effects on gene activation and cellular phenotype (Leonard and Shea 1998).

1.9 SOCS3: REGULATORS OF T CELL ACTIVATION

The suppressors of cytokine signaling (SOCS) family of proteins act as intracellular inhibitors of several cytokine signal transduction pathways. Their expression is induced by cytokine activation of the Janus kinases signal transducer and activator of transcription (JAK/STAT) pathway. The SOCS proteins are of particular interest because of their critical roles in regulating cytokine and growth factor signalling (Chen et al 2000, Alexander 2002). Recent studies show that SOCS proteins interact with proteins crucial to T cell activation (e.g. ZAP70, Gfi-1, and calcineurin) suggesting that they may play a role in the regulation of essential aspects of the T-helper cell activation program.
SOCS are a newly described family of intracellular, cytokine-inducible, negative feedback regulators that target cytokine receptors and cytoplasmic signalling adaptor molecules (Chen et al 2000, Alexander 2002). In addition to functioning in a classical feedback regulatory loop, SOCS proteins can also inhibit responses to cytokines that are different from those that induce their expression. Their inhibitory effects are derived from direct interaction of the SOCS proteins with cytokine receptors and/or Janus kinases, thereby preventing recruitment of signal transducers and activators of transcription to the signalling complex (Yasukawa et al 2000).

Thus Suppressors of Cytokine Signaling (SOCS) have been implicated in regulation of T cell activation and cytokine-mediated differentiation of T-helper cells. SOCS1, SOCS2, SOCS3, CIS (cytokine-induced SH2 protein) genes are constitutively expressed in naïve T-helper cells, with SOCS3 being the most abundant. Antigen-stimulation of naïve T-helper cells down regulates SOCS3 expression and concomitantly up regulates SOCS1, SOCS2, CIS genes transcription, suggesting that SOCS genes are differentially regulated by T-cell activation. SOCS3 is highly expressed in Th2 cells (Egwuagu et al 2002), underscoring the involvement of SOCS proteins in T-cell development. The differentiation of naive T-helper into mature Ag-specific Th1 or Th2 phenotype is associated with preferential expression of SOCS1 and SOCS3, respectively (Egwuagu et al 2002).

Down regulation of SOCS3 expression after antigen-stimulation is subsequently followed by gradual increase in SOCS3 level and corresponding decline in IL-2 secretion. In fact, SOCS3 mRNA levels are inversely correlated with the amount of IL-2 secretion and proliferative responses of differentiating T-helper cells, suggesting mutually antagonistic effects of SOCS3 and IL-2 and feedback regulation of T cell activation by SOCS3.
Transient inhibition of SOCS3 by antigen-stimulation may therefore be essential in allowing activation of resting T cells. Forced over-expression of SOCS3 inhibits proliferation of T-helper cells.

1.10 DRUG DISCOVERY

Drug discovery is a complex, interdisciplinary pursuit, of chemistry, pharmacology, basic and clinical sciences which has benefited the humankind immensely. Most drugs, in the past, have been discovered either by identifying the active ingredient of traditional remedies or by serendipity. Modern drug discovery process involves understanding how disease and infections are controlled at the molecular and physiological level (Drews 2000, Ng 2004).

1.11 OVERVIEW OF NATURAL PRODUCTS IN DRUG DISCOVERY – HISTORY, PLANTS AS ABUNDANT SOURCE AND FUTURE

Natural products have played an important role in treating and preventing human diseases. Plants have formed the basis of sophisticated traditional medicine systems that have been in existence for thousands of years. The first records, written on clay tablets, are from Mesopotamia and date from about 2600 BC; amongst the approximately 1000 plant derived substances which they used were oils of Cedrus species (cedar) and Cupressus sempervirens (cypress), Glycyrrhiza glabra (licorice), Commiphora species (myrrh), and Papaver somniferum (poppy juice), all of which are still in use today for the treatment of ailments ranging from coughs and colds to parasitic infections and inflammation.

The Chinese Materia Medica has been extensively documented over the centuries, with the first record dating from about 1100 BC (Wu Shi Er
Bing Fang, containing 52 prescriptions), followed by works such as the Shennong Herbal (~100 BC; 365 drugs) and the Tang Herbal (659 AD; 850 drugs). Likewise, documentation of the Indian Ayurvedic system dates from about 1000 BC (Charaka; Sushruta and Samhitas with 341 and 516 drugs respectively), and this system formed the basis for the primary text of Tibetan medicine, Gyu-zhi during the eighth century AD (Dev 1999, Fallarino 1994).

Natural product medicines have come from various source materials including terrestrial plants, terrestrial microorganisms, marine organisms, and terrestrial vertebrates and invertebrates (Newman et al 2000). The importance of natural products in modern medicine has been discussed in recent reviews and reports (Newman et al 2003, Newman and Cragg 2007, Koehn and Carter 2005, Paterson and Anderson 2005, Balunas et al 2005). The value of natural products in this regard can be assessed using 3 criteria: (1) the rate of introduction of new chemical entities of wide structural diversity, and their subsequent use as templates for semisynthetic and total synthetic modification, (2) the number of diseases treated or prevented by these substances, and (3) their frequency of use in the treatment of disease.

An analysis of the approval of the drugs developed between 2000 and 2006 showed that natural products or natural product-derived drugs comprised ~50% of all new chemical entities (NCEs) launched onto the market (Newman and Cragg 2007). In addition, 30% of these NCEs were synthetic or natural mimic compounds, based on the study of pharmacophores related to natural products (Newman and Cragg 2007). This suggests that natural products are important sources for new drugs and are also good lead compounds suitable for further modification during drug development. The large proportion of natural products in drug discovery has stemmed from the diverse structures and the intricate carbon skeletons of natural products. Since secondary metabolites from natural sources have been elaborated within
living systems, they are often perceived as showing more “drug-likeness and biological friendliness than totally synthetic molecules,” (Koehn and Carter 2005) making them good candidates for further drug development (Balunas et al 2005, Drahl et al 2005).

Examination of medical indications by source of compounds has demonstrated that natural products and related drugs are used to treat 87% of all categorised human diseases, including as antibacterial, anticancer, anticoagulant, anti parasitic and immunosuppressant agents, among others (Newman et al 2003). In the case of antibacterial agents, natural products have made significant contributions as either direct treatments or templates for synthetic modification. Of the 90 drugs of that type that were approved worldwide from 1982 to 2002, ~79% can be traced to a natural product origin (Newman et al 2003).

Frequency of use of natural products in the treatment and/or prevention of disease can be measured by the number and/or economic value of prescriptions, from which the extent of preference and/or effectiveness of drugs can be estimated indirectly. According to a study by Grifo and colleagues (Grifo et al 1997), 84 of a representative 150 prescription drugs in the United States fell into the category of natural products and related drugs. They were prescribed predominantly as anti-allergy/pulmonary/respiratory agents, analgesics, cardiovascular drugs, and for infectious diseases. Another study found that natural products or related substances accounted for 40%, 24%, and 26%, respectively, of the top 35 worldwide ethical drug sales from 2000 2001 and 2002 (Butler 2004). Of these natural product-based drugs, paclitaxel (ranked at 25 in 2000), a plant-derived anticancer drug, had sales of $1.6 billion in 2000 (Thayer 2003, Oberlies and Kroll 2004). The sales of 2 categories of plant-derived cancer chemotherapeutic agents were responsible for approximately one third of the total anticancer drug sales worldwide, or
just under $3 billion dollars in 2002, namely, the taxanes, paclitaxel and docetaxel, and the camptothecin derivatives, irinotecan and topotecan (Thayer 2003, Oberlies and Kroll 2004).

Historical experiences with plants as therapeutic tools have helped to introduce single chemical entities in modern medicine. Plants, especially those with ethno pharmacological uses, have been the primary sources of medicines for early drug discovery. In fact, a recent analysis showed that the uses of 80% of 122 plant-derived drugs were related to their original ethno pharmacological purposes (Fabricant and Farnsworth 2001). Current drug discovery from medicinal plants has mainly relied on bioactivity-guided isolation methods, which, for example, have led to discoveries of the important anticancer agents, paclitaxel from *Taxus brevifolia* and camptothecin from *Camptotheca acuminata* (Kinghorn 1994).

The data shown in Figure 1.4 categorises natural sources in the following way: Biological (B) - a peptide or protein isolated from an organism or cell line, Natural product (N), Natural product derivative (ND) -

![Figure 1.4](image-url)  
**Figure 1.4** Pharmaceuticals containing natural products Adapted from Newman et al (2007)
derived from a natural product usually with some semi-synthetic modifications, Synthetic drug (S), Synthetic drug with a natural product pharmacophore, vaccine (S*) and Vaccines (V) (Newman and Cragg 2007).

1.12 DRUG DISCOVERY IN SILICO

Drug discovery traditionally has been understood in two steps: a) target identification and screen development and b) drug lead identification and optimization. The first step involves understanding the biological basis for a disease, including the natural pathways and biochemical reactions, along with the deviations that cause the disease. Post – genomics era has witnessed an explosion of information. Researches want to identify the functionality of the protein encoded by a gene in the vast genome of an organism. This has led to a boom in structural genomics. Drug discovery has also been party to this information explosion. Previous, conventional drug discovery methods have been superceded. Modern day drug discovery process has been revamped to suit the dearth of information available to the researcher. Advancement in computational power has enabled the researcher to screen millions of structures from a library in silico against the protein target of interest. This has ensured a rapid screening method to pick up only the most relevant drug candidate, saving precious time.

1.12.1 Molecular Modelling

Docking can be thought of as a combination of spatial sampling methods with energy scoring functions, that search over many possible interactions between a ligand and a receptor molecule in order to identify a set of ligand poses that represent local minimum energy positions of the ligand. If the ligand pose sampling is adequate, and energy scoring function is sufficiently accurate then the global minimum energy position of the ligand in the receptor can be selected from the set of local energy minima. While
docking is most often applied to systems of small molecule ligands and protein receptors it has also been used to dock proteins to proteins, nucleic acids to proteins, and small molecules to nucleic acids.

There are a number of well known docking systems such as DOCK, FLEEx, GOLD, GLIDE, and these vary in the method of evaluating and optimizing the predicted binding affinity. Many docking systems use molecular mechanics force field methodologies to estimate the binding affinity. These methodologies attempt to model the short range and long range forces between a target and a small molecule using field representations. The types of interaction often considered in docking systems include electrostatic contributions, steric interactions, hydrogen bonding stabilization and hydrophobic effects. Other important considerations for docking include the effect of solvation and the flexibility of the protein itself.

One commonly used system is AUTODOCK. This program uses a grid based technique in which the interaction energies for the atoms in the small molecule are precalculated on the points of a grid. This process simplifies the calculation of the energy estimate of small molecule in a particular position. The grids are determined by standard molecular mechanics force field methods. The position and conformation of the small molecules are adjusted using a hybrid genetic algorithm to sample over the feasible conformations and positions of the ligand relative to the protein.

1.13 MEDICINAL PROPERTIES AND ETHNOBOTANICAL INFORMATION OF Calotropis procera

*Calotropis procera* (Figure 1.5) is a wild plant growing in tropical regions. In Indian traditional medicine, this plant has been advocated for a variety of disease conditions including leprosy, ulcers, tumours and piles. Leaves and roots of this plant have been used to relive pain under different
conditions like toothache, joint pain and migraine (Wealth of India 1992) and skin disease, rheumatism and aches (Kirtikar and Basu 1935).

Traditionally in rural areas, every constituent part of the plant is used in the treatment of various ailments. The milky latex is used in the treatment of leprosy, eczema, inflammation, cutaneous infections, syphilis, malarial and low hectic fevers, and also as a botifacient (Singh 1995, Shah 1982, Kumar and Basu 1994, Lindley 1981, Khory and Kathrak 1981, Ayoub and Suendsen 1981) The leaves have been used as an anti-inflammatory and anti-microbial agent in rheumatism (Sebastian and Bhandari 1984, Kumar and Chauhan 1992). The roots of *Calotropis procera* are used as a hepatoprotective agents, against colds and coughs, syphilis and elephantiasis, as an anti-inflammatory, analgesic, antimalarial and antimicrobial agent (Basu and Nagachaudhari 1991, Singh and Ali 1994). The flowers of the plant have been found to be cytostatic, abortifacient, antimalarial, in asthma and piles (Smit et al 1995).

The milky latex of *C. procera* which is strongly purgative and caustic is well known for its medicinal properties. The dry latex of *C. procera* a potent anti-inflammatory agent (Singh et al 2000) has been also evaluated for anti-diarrhoeal activity. The crude latex of *C. procera* is known to exhibit intense inflammation by local administration, associated with increase in capillary permeability, oedema and cellular influx (Shivkar and Kumar 2003). Interleukin-1β inhibits paw oedema induced by local administration of latex extracts of *C. procera*. The plant has been evaluated for its antioxidant and anti-hyperglycemic effects against alloxan induced diabetes in rats (Roy et al 2005).
The plant Calotropis is rich in Alkaloids, flavonoids, tannins, sterols, triterpenes, saponins, cardiac glycosides (Deepak 1995). Compounds derived from the plant have been found to have emeto-cathartic and digitalic properties. The principal active medicinals are asclepin, calactin and mudarin. (Figure 1.6) Other compounds have been found to have bactericidal and vermicidal properties. The latex contains a proteolytic enzyme called caloptropaine.
Figure 1.6 Some well known drug molecules isolated from *Calotropis procera*

1.14 OBJECTIVES

The objective of this study is based on the concept of phytochemistry, ethnobotany, cellular biology, and computational biology. In brief, the anti-inflammatory effect of *Calotropis procera* (Asclepiadaceae) leaf was assessed on human cell models by analysing the cellular and molecular targets that are affected in inflammatory disorders.

The following are the major objectives of this study

- Screen for anti-inflammatory potential of *Calotropis procera* on T-cells (JURKAT) and macrophages (RAW 264.7) using specific *in vitro* screening bioassays.
- Identify, isolate and structurally characterize the bioactive lead molecule.
Elucidate the probable mechanism of action of the bioactive lead molecule by studying the major signalling events involved in inflammation.

Understand, using molecular docking program, the binding interactions of the isolated bioactive molecule to specific protein target, namely COX-2.