An overview on inflammation and anti-inflammatory drugs

The bulk of our most lethal diseases have a common underlying cause: ‘persistent inflammation’. Inflammation is an overactive reaction of the natural immune system function that results in cell and tissue destruction. This persistent inflammation is triggered by our industrial lifestyles, including exposure to chemicals, synthetic food ingredients, pollution and processed foods. Earlier in the fifties and early sixties, the treatment of inflammation revolved around high dose aspirin, other non-steroidal anti-inflammatory drugs and corticosteroids which provided symptomatic relief but did not affect the underlying disease process. At the same time these drugs caused side-effects on long term use (Kumar and Marwaha, 2003).

Later a wide spread interest grew among researchers for exploring biologically active phytochemicals with anti-inflammatory property. The structural diversity and relatively low toxic nature of phytochemicals prompted them to investigate the anti-inflammatory activity of these compounds. The usage of phytochemicals dated back to the period of Greeks and Romans when the bark of ‘willow tree’ was used to treat inflammation. The ancient Greek physician Hippocrates wrote about its medicinal properties in the 5th century BC. In the mid-eighteenth century, salicylate-rich willow bark extract became recognized for its specific effects on fever, pain and inflammation. By the nineteenth century pharmacists were experimenting with and prescribing a variety of chemicals related to salicylic acid, the active component of willow extract. The discovery of the first anti-inflammatory drug ‘acetyl salicylic acid’ or ‘aspirin’ in 1899 was based on the analgesic and antipyretic properties of the bark of this willow-tree. Today, many phytochemicals dispensed in the form of crude drugs such as tinctures, teas, poultices, powders and other herbal formulations serve as the basis of novel drug discovery. A number of reviews are available on inflammation and
the emerging role of phytochemicals in countering it (Gilroy et al., 2004; Talhouk et al., 2007). A brief review on inflammation, particularly in relation to rheumatoid arthritis and wide range of therapies aiming against this auto immune disease is given below:

1.1 Inflammation

Inflammation is body’s response to disturbed homeostasis caused by infection, injury, post ischemia, toxins, allergy, autoimmune injury or trauma resulting in systemic and local effects. Inflammation is characterized by

(a) vasodilation of the local blood vessels with consequent excess local blood flow;
(b) increased permeability of the capillaries allowing leakage of large quantities of fluid into the interstitial space; (c) clotting of the fluid into the interstitial space because of excessive amounts of fibrinogen and other proteins leaking from the capillaries; (d) migration of large numbers of granulocytes and monocytes into the tissue and (e) swelling (edema) of the tissue cells. Some of the tissue products that cause these reactions are histamine, bradykinin, serotonin, prostaglandins, reaction products of the complement system, reaction products of the blood-clotting system and lymphokines released by sensitized cells (fig. 1.1) (Guyton and Hall, 2000).

Figure 1.1 Key events in inflammatory process: Mast cells release histamine that causes vasodilation resulting in exudation of plasma proteins activating complement cascade. Polymorphonuclear leukocytes phagocytose microbes. Lymphocytes elicit immune response. Monocytes differentiate to macrophages; eliminate microbes and releases pro-inflammatory cytokines. Acellular biochemical cascade systems like plasma proteins, complement system, coagulation and fibrinolysis systems act in parallel to initiate and propagate the inflammatory response. Cellular and acellular biochemical processes act in concert to initiate repair mechanisms.
Each type of stimulus provokes a characteristic pattern of response. The Roman encyclopedist Celsus in the 1st century AD macroscopically described the four cardinal signs of inflammation: rubor (redness), tumor (swelling or edema), calor (heat) and dolor (pain). Later Virchow added the fifth sign of inflammation function laesa (loss of function) (Shibata et al., 1989; Chandrasoma and Taylor, 2005). These characteristic inflammatory responses occur in the three distinct phases, each apparently mediated by different mechanisms: (a) an acute transient phase characterized by local vasodilation and increased capillary permeability; (b) a delayed, sub-acute phase, most prominently characterized by infiltration of leukocytes and phagocytic cells and (c) a chronic proliferative phase, in which tissue degeneration and fibrosis occur (Gallin et al., 1999).

An inflammatory response can be acute or chronic depending on the cell types and mediators participating in the reaction and on the duration of condition. Acute inflammation is a short-lived process (appearing in a few minutes or hours) which results when the causative agent is not neutralized by the host and/or if there is a failure in resolving inflammation (Shibata et al., 1989). This process is a limited beneficial process, particularly during infectious challenge. A hallmark of acute inflammation is that initially the leukocyte infiltrate is mostly neutrophilic, and later (after 24 to 48 hours) turns monocytic (Ryan and Majno, 1977).

In contrast to short-lived acute inflammation, chronic inflammation is a prolonged process. It is histologically associated with the presence of mononuclear cells such as macrophages and lymphocytes (Ryan and Majno, 1977). Chronic inflammation is initiated by the development of an immune response to an endogenous antigen (Dumonde and Glynn, 1962). The antigenic stimulus activates repair mechanisms causing in-growth from the surrounding connective tissue resulting in formation of a granulation tissue. With time, the fibroblasts lay down collagen, the capillaries disappear and an avascular area of fibrosis or scar results. In rheumatoid arthritis, repeated bouts of acute inflammation occur followed by granulomatous tissue formation, angiogenesis, fibrosis and scar formation (Cotran et al. 1999).
A hallmark of chronic inflammation is the increased expression of adhesion molecules like intracellular adhesion molecule-1 (ICAM-1), E-selectin and vascular cell adhesion molecule-1 (VCAM-1) on endothelium surface (Pitzalis et al., 1994). These adhesion molecules facilitate the ‘rolling’ and adhesion of inflammatory phagocytic cells to the vascular endothelium at vessel sites adjacent to sites of inflammation (fig. 1.2). The phagocytic cells then migrate through vessel wall via the process of diapedesis, get activated and release mediators that perpetuates inflammation (Winyard, 2003).

![Figure 1.2 Inflammatory response](image)

**Figure 1.2 Inflammatory response:** Leukocyte interactions with vascular endothelium at sites of inflammation can be dynamically regulated by activation-dependent adhesion molecules. During this process there is involvement of multiple members of the selectin, integrin, and immunoglobulin gene families which facilitates initial attachment (rolling), stable adhesion (arrest), spreading and ultimate diapedesis.

### 1.2 Cellular components of inflammation

The cellular components of inflammation include neutrophils, dendritic cells, monocytes, macrophages, eosinophils, mast cells, lymphocytes and platelets. All these cells are capable of producing assorted array of cytokines, cytotoxic mediators including reactive oxygen species, serine and cysteine proteases, matrix metalloproteinases (MMPs), membrane perforating agents and soluble mediators of inflammation like prostaglandins, leukotrienes, nitric oxide and pro-inflammatory cytokines (Kuper et al., 2000; Wahl and Kleinman, 1998).
During inflammation, platelet activation initiates inflammatory response by secreting molecules such as arachidonic acid metabolites, heparin, serotonin, thrombin, coagulation factors, platelet derived growth factor (PDGF), transforming growth factors like TGF-α and TGF-β, fibroblast growth factor (FGF), enzymes, proteinase inhibitors etc. This is followed by granulation of tissue which is facilitated by chemotaxis of neutrophils, monocytes and fibroblast as well as synthesis of new extracellular matrix and angiogenesis (Coussens and Werb, 2002).

Neutrophils are the first recruited effectors of the acute inflammatory response. They are capable of producing a wide array of pro-inflammatory cytokines such as tumor necrosis factor -α (TNF-α) (Feiken et al., 1995) and interleukins like IL-1α and IL-1β necessary for other effector cell recruitment, activation and response including adhesion of leukocyte to the vascular endothelium, expression of matrix metalloproteinases and keratinocyte growth factor by fibroblasts (Chedid et al., 1994; Brigati et al., 2002).

Monocytes are also key players in inflammatory response. Guided by chemotactic factors like TGF-β, PDGF, chemokines like monocyte chemoattractant protein-1, -2 and -3, macrophage inflammatory proteins like MIP-1α and MIP-1β and cytokines like IL-1β and TNF-α, the monocytes migrate to the site of inflammation and gets converted to macrophages. Macrophages on activation produce growth factors and cytokines like TGF-β, PDGF, FGF, insulin like growth factor IGF-1 and -2, TNF-α and IL-1. All these products affect other cells in their focal microenvironment. Macrophages also induce tissue remodeling and stimulate proteolytic enzymes like MMPs and modulate angiogenesis by local production of thrombospondin (Di Pietro et al., 1995).

Mast cells are also involved in inflammation. There are two types of mast cells: connective tissue type mast cells (CTMC) and mucosal type mast cells (MTMC). Derived from the bone marrow, these cells reside in connective or mucosal tissues and synthesize inflammatory mediators like histamine, cytokines, eicosanoids (CTMC release PGD<sub>2</sub> in response to IgE and antigen, whereas MTMC preferentially release LTC₄ on stimulation by IgE and antigen), MMPs, tryptase.
and other serine proteases within granules and degranulate them on activation. Mediators like histamine, eicosanoids and tryptase cause vasodilation and extravasation of fluid.

The natural killer cells (NK cells) are bone marrow derived lymphocyte with ability to lyze certain tumour cells. They are important part of the innate immune system. These cells mediate cytotoxicity by release of perforin and granzymes and through cytokine production especially interferon-γ.

B cells play role in mediating immune response. In addition to their ability to produce antibodies, they present antigen to T cells. They also produce a variety of cytokines like IL-1, IL-2, IL-4, IL-6, IL-10, IL-12, IL-13, IL-16, INF-γ, lymphotoxin α and β, TGF-β and TNF-α. Distinct subset of cytokine secreting B cells (Be 1 and Be 2) secrete type I cytokine (INF-γ, IL-12) or type II cytokines (IL-2, IL-4, IL-6) respectively (Harris et al., 2000; Lund et al., 2005) and modulates inflammatory response.

1.3 Mediators of inflammation

A substance that causes inflammation through a specific receptor is called mediator of inflammation. Both endogenous and exogenous substances like proteins, lipids, histamine, serotonin, nitric oxide, interleukins, tumor necrosis factors etc. may act as mediators. These mediators generate systemic responses to the inflammatory process like fever, hypotension, leukocytosis, cachexia and synthesis of acute phase proteins. Mediators of inflammation belong to the following classes:

- Lipid mediators (prostaglandins, leukotrienes and platelet activation factor)
- Nitric oxide
- Cytokines
- Chemokines
- Oxygen-derived free radicals
- The clotting system products (plasmin, fibrinopeptides)
- Fibrinolytic system products (fibrin)
- Kinins (bradykinin)
- Vasoactive amines (histamine and 5-hydroxytryptamine)
- Substance P
- Complement system by-products
- Cell-adhesion molecules

1.3.1 Lipid mediators

The major constituents of cell membranes are phospholipids. During inflammation, cellular phospholipases like phospholipase A₂ and C degrade phospholipids to arachidonic acid. Arachidonic acid has a short half-life and is metabolized by two routes, the cyclooxygenase and lipoxygenase pathways (fig. 1.3).

![Figure 1.3 Cyclooxygenase and lipoxygenase pathway](image)

1.3.1.1 Cyclooxygenases and prostaglandins

Cyclooxygenases (COX) or prostaglandin endoperoxide H synthases are enzymes that catalyze a bisoxygenase reaction leading to the production of important biological mediators called prostanoids which includes prostaglandins, prostacyclins and thromboxanes. COX has two catalytic activities, a
cyclooxygenase activity in which arachidonic acid gets converted to prostaglandin G2 (PGG2) and a peroxidase activity in which PGG2 undergoes a two electron reduction to PGH2 which is then processed to various classes of bioactive lipids like thromboxanes, PGF2α, PGD2, PGI2 and PGE2 (fig. 1.4).

![Prostaglandin E2 biosynthetic pathway](image)

Figure 1.4 The prostaglandin E2 biosynthetic pathway. DP, PGD2 receptor; EP, PGE2 receptor; FP, PGF2α receptor; mPGES, microsomal prostaglandin E synthase; PG, prostaglandin; TP, thromboxane A2 receptor; TXA2, thromboxane A2.

Three isoforms of COXs have been identified: COX-1, COX-2 and COX-3. COX-1 is expressed in mammalian cells, particularly in the endothelium, platelets and kidneys under physiological conditions where it is involved in homeostatic process. The inducible form COX-2 is expressed under pathological conditions. COX-2 expression in cells can also be induced by inflammatory stimuli like IL-1, IL-2, TNF-α, LPS, GM-CSF, TGF-β, phorbol myristate, cigarette smoke etc. (Katori and Majima, 2000; Pei et al., 2009). Moses et al. (2009) stated that Toll
like receptor-4 has role in stimulating COX-2 expression and PGE₂ production. Constitutive expression of COX-2 has also been reported especially in brain and kidney (Smith et al., 1996; Jouzeau et al., 1997).

### Table 1.1 Physiological/pathological functions of COX-1 and COX-2

<table>
<thead>
<tr>
<th>PHYSIOLOGICAL/PATHOLOGICAL PROCESS</th>
<th>COX-1</th>
<th>COX-2</th>
<th>PGs INVOLVED</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovulation</td>
<td>Not essential</td>
<td>Essential</td>
<td>PGE₂</td>
</tr>
<tr>
<td>Implantation</td>
<td>Not essential</td>
<td>Essential</td>
<td>PGI₂</td>
</tr>
<tr>
<td>Parturition</td>
<td>Essential</td>
<td>Compensatory</td>
<td>PGF₂α</td>
</tr>
<tr>
<td>Inflammatory signs</td>
<td>Essential in some kind of inflammation</td>
<td>Essential</td>
<td>Not specified</td>
</tr>
<tr>
<td>Inflammation resolution</td>
<td>Not essential</td>
<td>Essential</td>
<td>PGD₂, 15-deoxy PGI₂</td>
</tr>
<tr>
<td>Platelet aggregation</td>
<td>Essential</td>
<td>No role</td>
<td>TXA₂</td>
</tr>
<tr>
<td>Perinatal kidney development</td>
<td>Not essential</td>
<td>Essential</td>
<td>Not specified</td>
</tr>
<tr>
<td>Kidney functions</td>
<td>Essential</td>
<td>Essential</td>
<td>PGE₂</td>
</tr>
<tr>
<td>Ductus arteriosus remodeling</td>
<td>Compensatory</td>
<td>Essential</td>
<td>TXA₂/PGH₂</td>
</tr>
<tr>
<td>T cell development</td>
<td>Stage specific</td>
<td>Stage specific</td>
<td>PGE₂</td>
</tr>
<tr>
<td>GI mucosa protection</td>
<td>Both essential under inflammatory condition</td>
<td>-</td>
<td>Several PGs</td>
</tr>
<tr>
<td>Gastric ulceration</td>
<td>Inhibition of both isoforms necessary</td>
<td>-</td>
<td>Several PGs</td>
</tr>
<tr>
<td>Ulcer healing</td>
<td>Not essential</td>
<td>Essential</td>
<td>PGE₂</td>
</tr>
<tr>
<td>Intestinal cancer</td>
<td>Essential</td>
<td>Essential</td>
<td>Several PGs</td>
</tr>
<tr>
<td>Crypt stem cell survival</td>
<td>Essential</td>
<td>Compensatory</td>
<td>PGE₂</td>
</tr>
</tbody>
</table>

(Martell-Pelletier et al., 2003)

COX-1 and COX-2 isozymes are 60% homologous. These isozymes are encoded by two different genes. The difference in specificity between the two isoforms is due to a single amino acid difference between COX-1 and COX-2 hydrophobic channels. COX-2 gene contains a TATA box which is absent in COX-1 (Smith et al., 1996). COX-2 promoter also contains several consensus sequences for transcription factors like cAMP element (CRE), NF-IL6 element,
κB, interferon stimulated responsive elements (ISRE), interferon regulatory factor (IRF) elements and glucocorticoid response element (Wadleigh et al., 2000). Other differences between COX-1 and COX-2 are differences in utilization of arachidonic acid substrate pools and mRNA stability (Kutchera et al., 1996; Reddy and Herschman, 1999). COX-3 is a splice variant of COX-1 which retains one intron and has a frameshift mutation (COX-1b or COX-1 variant) (Chandrasekharan et al., 2002).

Prostaglandins (PGs), the end-product of COX pathway, act as an autocoid playing role similar to hormones. PGs, once generated, send signals to adjacent cells in a paracrine fashion, or to the generated cells in an autocrine fashion. PGE$_2$ enhances vascular permeability, increases sensitivity to pain, stimulates leukocyte cAMP, suppresses the release of mediators by mast cells, lymphocytes and phagocytes and also has effect on bone formation (Crunkhorn and Willis, 1971; Raisz and Fall, 1990). PGE$_2$ and PGI$_2$ potentiate the activity of histamine and bradykinin and increases vascular permeability of blood vessels. These prostaglandins also sensitize sensory nerve terminals and potentiate pain producing action of bradykinin. Another characteristic feature of PGE$_2$ is that it inhibits release of TNF-α and IL-1 under a negative feed back mechanism and at the same time enhances release of IL-6 by its generation from the same cells in a positive feed back loop (Utsunomiya et al., 1994). PGE$_2$ also suppresses norepinephrine release from peripheral sympathetic nerve terminals (Hedqvist, 1974). Thus PGs are short-term modifiers of cellular function through its switch-like on-off actions.

**Regulation of COX expression**

DNA binding proteins such as interferon regulatory factors (IRF-1, IRF-2), NF-IL-6, c/EBPβ and STAT 1α possess important roles in initiating transcription of COX-2 gene. IRF-1 has stimulatory effect whereas IRF-2 has an inhibitory effect. The presence of cyclic adenosine monophosphate (cAMP) response element (CRE) in COX-2 promoter allows COX-2 expression to be directly regulated by feedback mechanism. Post-transcriptional factors also play key role in the expression of COX-2 (Rimarachin et al., 1994; Jouzeau et al., 1997).
1.3.1.2 Lipoxygenases and leukotrienes

Lipoxygenases (LOXs) are a family of iron-containing enzymes that catalyze the dioxygenation of polyunsaturated fatty acids in lipids containing a cis-1,4- pentadiene structure to produce leukotrienes. Leukotrienes (LTs) are lipid signaling molecules derived from arachidonic acid (AA) that initiate and amplify innate and adaptive immune responses by regulating the recruitment and activation of leukocytes in inflamed tissues. Mammalian lipoxygenases are of three types: 5, -12 and -15 lipoxygenase. 5-LOX catalyze the synthesis of leukotrienes from arachidonic acid. The catalytic mechanism involves the insertion of an oxygen moiety at a specific position in the arachidonic acid backbone. The lipoxygenase pathway is active in leukocytes, including mast cells, eosinophils, neutrophils, monocytes and basophils.

![Diagram of 5-LOX and 15-LOX pathway](image)

**Figure 1.5 5-LOX and 15-LOX pathway**

The initial enzymatic steps in leukotriene synthesis occur at the nuclear envelope. Cytosolic phospholipase A₂ translocates from the cytoplasm to selectively hydrolyze nuclear envelope phospholipids, releasing free arachidonate. The released arachidonic acid is donated by the 5-lipoxygenase activating protein (FLAP) to 5-lipoxygenase (5-LOX). 5-LOX uses FLAP to convert arachidonic
acid into 5-hydroperoxyeicosatetraenoic acid (5-HPETE), which spontaneously gets reduced to 5-hydroxyeicosatetraenoic acid (5-HETE) and then to leukotriene A₄ (LTA₄), an unstable epoxide (Lipkowitz et al. 2001) (fig. 1.5). In neutrophils and monocytes, LTA₄ is then converted to the dihydroxy acid leukotriene LTB₄ which is a potent chemoattractant for phagocytes and an important component of body defense mechanism against infection (Werz et al., 1997). In resting human and rat peripheral blood neutrophils, 5-LOX is localized in the cytoplasm and in rat basophilic leukemia cells and human alveolar macrophages it is found predominantly in the nucleus. Immunofluorescence microscopy studies demonstrate that both cytoplasmic and nuclear 5-LOX move to the nuclear envelope following cell activation (Golden et al., 1998) (fig. 1.6).

LTB₄ produced by 5-LOX is generated mainly by cell types that drive inflammatory processes like macrophages and neutrophils (Hubbard et al., 1991). The main biological functions of leukotriene B₄ (LTB₄) are recruitment and activation of inflammatory cells like neutrophils, macrophages, monocytes, eosinophils and lymphocytes (Ford-Hutchinson, 1990). LTB₄ increases vascular permeability, induces the expression of adhesion molecules like Mac-1 (CD11b/CD18) on polymorphonuclear leucocytes for leukocyte adherence to endothelial cells (Morgan et al., 1995) and causes neutrophils to accumulate rapidly in the affected tissue (Camp et al., 1983).
Introduction

LTB₄ contributes to accumulation of not only of neutrophils but also of macrophages, T lymphocytes and eosinophils at the site of inflammation. LTs exerts effects on various immunological phenomena such as release of cytokines (interleukin-1, tumour necrosis factor (TNF), interferon-γ and interleukin-2) and matrix metalloproteinases-2, -3, and -9 (Rola-Pleszczynski et al., 1987; Leppert et al., 1995). LTB₄ augments IL-6 production in human monocytes by increasing IL-6 gene transcription, mRNA stabilization and activation of NF-κB (Brach et al., 1992; Rola-Pleszczynski and Stankova, 1992). LTB₄ is also an important mediator of pain. Injection of LTB₄ into a rat paw results in a prolonged, neutrophil-dependent hyperalgesic reaction, which is associated with a sustained reduction in the nociceptive pressure threshold (Levine et al., 1984).

In cells that express LTC₄ synthase, such as mast cells and eosinophils, LTA₄ is conjugated with the tripeptide glutathione to form cysteinyl-leukotrienes, LTC₄. Outside the cell, LTC₄ can be converted by ubiquitous enzymes to form successively LTD₄ and LTE₄, which retain biological activity. LTC₄, D₄ and E₄ are potent mediators of bronchospasm in chronic inflammatory disease like asthma (Hay et al., 1995). Experimental trials demonstrated that cysteinyl leukotrienes participate in the damage of gastric mucosa by inducing mucosal microvascular injury, gastric vessel vasoconstriction, secretion of gastric acid and production of pro-inflammatory cytokines (Peskar et al., 1991).

Another lipoxygenase, 15-LOX is a lipid-peroxidizing enzyme mainly expressed in airway epithelial cells, eosinophils, reticulocytes and macrophages. In humans, 15-LOX exists as two different enzymes with different cell localizations and product profiles (Andersson et al., 2006). 15-LOX-1 converts arachidonic acid to an unstable intermediate, 15-hydroperoxyeicosatetraenoic acid, which can be further converted to 15-hydroxyeicosatetraenoic acid (15-HETE). The 15-LOX-1 enzyme has pro-inflammatory actions, with high levels of 15-HETE reported in sputum of asthmatic patients along with increased macrophage 15-LOX-1 mRNA expression (Profita et al., 2000). Experimental trials demonstrated that 15-LOX-1 expression can be induced by IL-13 in human blood monocytes (Deleuran et al., 1995) and by IL-4 in monocytes, alveolar macrophages, dendritic cells, mast cells
and rheumatoid arthritis synovial cells where it initiates inflammatory processes (Nassar et al., 1994).

**Regulation of leukotriene biosynthesis**

In cells, inhibition of LT synthesis is achieved by direct 5-LOX inhibition which prevents conversion of arachidonic acid to LTA₄ and by inhibition of 5-LOX activating protein (FLAP). FLAP, a nuclear membrane-bound peptide functions as an arachidonate transfer molecule which is required for 5-LOX activity in intact cells (Miller et al., 1990; Mancini et al., 1993). Rouzer et al. (1990) reported that FLAP inhibition prevented translocation of 5-LOX from cytosol to membrane and suppressed 5-LOX inhibition.

1.3.2 Nitric oxide synthase and nitric oxide

Nitric oxide synthase produces NO by catalyzing a five-electron oxidation of a guanidino nitrogen of L-arginine.

![Figure 1.7 Conversion of L-arginine to L-citrulline](image)

Nitric oxide synthases are present among eukaryotic enzymes as dimeric, calmodulin-dependent hemoprotein that combine reductase and oxygenase catalytic domains in one dimer, bears both flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) and carries out a 5'-electron oxidation of non-aromatic amino acid L-arginine with the aid of tetrahydrobiopterin. Three forms of nitric oxide synthase (NOS) encoded by different genes are recognized. They are:

- NOS-1 / neuronal or nNOS, a constitutive enzyme
- NOS-2 / inducible or iNOS, inducible enzyme
- NOS-3 / endothelial or eNOS, constitutive enzyme
Introduction

All three isoforms share a carboxyl-terminal reductase domain and also share an amino-terminal oxygenase domain containing a heme prosthetic group, which is linked in the middle of the protein to a calmodulin-binding domain. Binding of calmodulin acts as a molecular switch to enable electron flow from flavin prosthetic groups in the reductase domain to heme. This facilitates the conversion of oxygen and L-arginine to NO and L-citrulline. The oxygenase domain of each NOS isoform also contains BH$_4$ prosthetic group, which is required for the efficient generation of NO.

The first nitric oxide synthase to be identified was nNOS found in neuronal tissue. The endothelial NOS, eNOS was the third to be identified. These enzymes were originally classified as ‘constitutively expressed’ and ‘Ca$^{2+}$ sensitive’ and are present in different cell types and its expression is regulated under specific physiological conditions. In nNOS and eNOS, the physiological concentrations of Ca$^{2+}$ in cells regulate the binding of calmodulin to the ‘latch domains’ in enzyme, thereby initiating electron transfer from the flavins to the heme moieties. In contrast, calmodulin is found tightly bound in inducible and Ca$^{2+}$ insensitive isoform (iNOS) even at a low intracellular Ca$^{2+}$ activity, acting essentially as a subunit of this isoform.

iNOS is predominantly produced by synoviocytes, chondrocytes, smooth muscle cells, hepatocytes and B-lymphocytes where its expression is induced by various cytokines like interferon-α, IL-1β, TNF-α and LPS (Clancy et al., 1998). Once synthesized, NO diffuses within the same cell or neighbouring cells and gets attached to the heme group of soluble guanylyl cyclase to generate cGMP and initiate smooth muscle relaxation (Murad, 2006). Activated cGMP then binds to specific target proteins including transcription factors, protein kinases and phosphodiesterases to elicit downstream effects. However, NO can also act in cGMP-independent manner, by directly modifying proteins or contributing to the oxidation of proteins and lipids, further increasing the complexity and number of potential roles for NO in normal and pathophysiological functions (Stamler et al., 2001).
Major functions of NO includes host resistance to tumors, microbes, regulation of blood pressure and vascular tone, neurotransmission, learning, neurotoxicity, carcinogenesis, control of cellular growth and differentiation (Nathan, 1992; Magrinat et al., 1992; Punjabi et al., 1992). Excessive production of NO is indicated in both acute and chronic inflammation (Clancy et al., 1998) (fig. 1.8).

**Figure 1.8 Physiological and pathological role of NO**

The cytotoxic effects of NO provide non-specific immunity not only for invading organisms, but also for the killing of cells (Hibbs et al., 1988). In the presence of oxygen, NO gets rapidly converted to nitrite and nitrate which are biologically inactive (Weinberg, 2000). When the concentration of superoxide radicals increases in the body, NO combines with superoxide radicals to generate biologically active peroxynitrite which contributes to tissue damage (Pacher et al., 2007). The local concentration of NO is an important determinant in cytotoxicity. The picomolar amounts of NO produced by cNOS isoforms are sufficient for intracellular signaling whereas micromolar concentrations generated by high output iNOS are microbicidal as well as pro-inflammatory and damaging to the surrounding cells and tissues (Farrell and Blake, 1996).

Complex cross-talks occur between the NOS and COX pathways. COX-2 expression is triggered by cytokines that induce iNOS. Endothelial cells, macrophages and chondrocytes produce NO and prostaglandins simultaneously in response to cytokines and other activators (Salvemini et al., 1996a; Amin et al.,
1997). The paracrine effects of these molecules are often similar and include the capacity to relax smooth muscles, inhibit platelet and neutrophil adhesion and inhibit neutrophil oxidant production. NO stimulates COX activity in RAW 264.7 murine macrophages, via reaction with the heme component which binds to the active site of the COX enzyme (Salvemini et al., 1996a). Conversely, reports also exist stating that NO attenuates PGE\(_2\) production and suppresses the expression of COX-2 protein (Amin et al., 1997).

**Regulation of nitric oxide synthase**

The promoter region upstream of 5’ region of iNOS gene contains numerous consenses sequences for DNA binding proteins like activating protein-1 (AP-1), NF-κB, TNF-element, INF-γ stimulable RE (ISRE), X-box, gamma activated sites (GAS), PU1/INF-γ element, NF-IL-6 and IFNγRE. AP-1(c-fos/c-jun) inhibits transcription whereas Interferon regulatory factor-1 (IRF-1), a protein factor that binds to γ-IRE DNA initiates iNOS transcription (Kamijo et al., 1994). Signal transducer and activator of transcription (STAT) factors are also important in activation of iNOS gene expression (Kleinert et al., 1998).

The inducible form iNOS is mainly regulated at the transcriptional level. 3’ untranslated region is reported to play a complex role in transcriptional upregulation of iNOS (Rodriguez-Pascual et al., 2000). At protein level, NOS is regulated by calmodulin binding, dimer formation, L-arginine (substrate) depletion, L-citrulline to L-arginine conversion (substrate recycling), tetrahydrobiopterin availability, NO interaction with NOS heme (end product inhibition), phosphorylation and subcellular localization. Important NOS cofactors include FAD, FMN, NADPH, tetrahydrobiopterin and calcium calmodulin (Weinberg, 2000).

**1.3.3 Cytokines**

Cytokines are major determinants of the state of cellular activation, the make up of the cellular infiltrate and the systemic responses to inflammation. Several cytokines secreted from activated immune cells and other inflammatory cells like monocytes and macrophages play an important role in the regulation of inflammatory responses by controlling proliferation, differentiation and effective function of immune cells (Murata et al., 1995) (fig. 1.10).
Cytokines are anti-inflammatory or pro-inflammatory in nature. Cytokines like IL-4, IL-10 and IL-13 are anti-inflammatory in nature (Feghali et al., 1997). IL-13 exhibits anti-inflammatory activity by inhibiting the production of lipopolysaccharide activated inflammatory cytokines such as IL-1β, TNF-α, IL-8 and IL-6 by human peripheral blood monocytes (Minty et al., 1993).

Pro-inflammatory cytokines like IL-1, TNF-α, IL-6, IL-11, IL-8 and chemokines like G-CSF and GM-CSF are involved in acute inflammation. The cytokines known to mediate chronic inflammatory processes can be divided into those participating in humoral inflammation, such as IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-13, TGF-β and those contributing to cellular inflammation such as IL-1, IL-2, IL-3, IL-4, IL-7, IL-9, IL-10, IL-12, interferons (IFNs), IFN-γ inducing factor (IGIF), TGF-β, TNF-α and TNF-β (fig. 1.9).

Two major cytokines that mediate inflammatory response are TNF-α and IL-6.
1.3.3.1 Tumor necrosis factor-alpha (TNF-α)

Tumor necrosis factor-alpha (TNF-α) is a pleiotropic inflammatory cytokine produced by activated monocytes, macrophages, fibroblasts, mast cells and natural killer cells. Human TNF α and β (also called lymphotoxin) are 17 and 25 kDa respectively. The gene encoding this cytokine is located on chromosome no. 6 in humans within the region of the major histocompatibility complex (Vilcek and Lee, 1991). TNF-α binds directly to its receptors TNFR-55 and TNFR-75 (expressed on all somatic cells) through cell-to-cell contact or undergoes cleavage and binds to its soluble form (Jones et al., 1989).

TNF-α possesses both growth stimulating as well as growth inhibitory properties and also possesses self regulatory properties. TNF-α not only induces neutrophil proliferation during inflammation, but also induces neutrophil apoptosis upon binding to the TNF-R55 receptor (Murray, et al., 1997). Tracey and Cerami (1990) suggested two beneficial functions of TNF-α which causes its continued expression. Firstly, low levels of the cytokine aids homeostasis by regulating the body’s circadian rhythm and secondly, low levels also promote remodeling or replacement of injured and senescent tissue by stimulating fibroblast growth. Additional beneficial functions of TNF-α include its role in the immune response to bacterial, certain fungal, viral and parasitic invasions as well as its role in the necrosis of specific tumors (Tracey and Cerami, 1990).

The pivotal role of TNF-α is that it acts as a key mediator in the local inflammatory immune response. TNF-α is an acute phase protein which initiates a cascade of cytokines and increases vascular permeability, thereby recruiting macrophage and neutrophils to a site of infection (Feghali et al., 1997). Without TNF- α, mice infected with gram negative bacteria experience septic shock (Janeway et al., 1999). TNF-α also induces fever by stimulation of PGE₂ synthesis by vascular endothelium of the hypothalamus or by directly inducing synthesis of IL-1 which elicits release of histamine from mast cells (Warren, 1990).

The pathological activities of TNF-α have attracted much attention. High levels of TNF-α correlate with an increased risk of mortality (Rink and Kirchner, 1996). TNF-α participates in both disorders of inflammatory and non inflammatory
Lipopolysaccharide, an endotoxin from bacteria cell walls is a potent stimulus for TNF-α synthesis (Tracey and Cerami, 1990). When TNF-α production increases to such an extent that it escapes the local infection, or when infection enters the bloodstream, sepsis ensues resulting in low blood volume, decreased blood pressure, hypoproteinanemia, neutropenia, neutrophilia, organ failure, myocardial suppression, dehydration, acute renal failure, respiratory arrest and finally death (Tracey and Cerami, 1990; Janeway et al., 1999).

1.3.3.2 Interleukin-6 (IL-6)

Interleukin-6 or IL-6 is a glycoprotein ranging from 21 to 28 kDa depending upon the degree of post-translational modification. The gene encoding IL-6 is located on chromosome 7 in humans. It is produced by various types of lymphoid and non-lymphoid cells, such as T cells, B cells, monocytes, fibroblasts, keratinocytes, endothelial cells, mesangial cells and several tumour cells (Hirano et al., 1990).

IL-6 plays a key role in acute phase response by stimulating the production of acute phase proteins (Gauldie et al., 1987). This pro-inflammatory cytokine elicits cellular immune responses to affected cells and mucosal humoral responses directed against reinfection (Kopf et al., 1994). IL-6 activates vascular endothelial cells, upregulates the expression of certain chemokines and adhesion molecules and facilitates leukocyte recruitment directly to the site of inflammation (Lipsky, 2006). IL-6 also plays role in mediating transition from acute to chronic inflammation (Kaplanski et al., 2003). IL-6 induces T cell growth and cytotoxic T cell differentiation by augmenting IL-2 receptor expression (Noma et al., 1986) and IL-2 production (Hibi et al., 1993). During hematopoiesis, IL-6 acts synergistically with IL-3 to support the formation of multilineage blast cell colonies, induces macrophage differentiation and initiates megakaryocyte differentiation (Yoshizaki et al., 1998).

Conventionally, IL-6 transmits signals by binding to its IL-6Rα on the cell surface and then associates with membrane bound gp130 (mgp130), a signal transducing membrane protein, resulting in dimerization of gp130 and signal transduction (Jones et al., 2005). The transmembrane form of IL-6R is confined to hepatocytes, monocytes/macrophages and some lymphocytes (Naka et al., 2002).
Introduction

where as the soluble form (sIL-6R) is found in serum and synovial fluids. IL-6 / sIL-6R / sgp130 inhibit IL-6 activity and limits systemic responses to IL-6 (Jostock et al., 2001). Many phytochemicals like epigallocatechin-3-gallate act by up regulating sgp-130 production and inhibiting IL-6 synthesis (Ahmad et al., 2008).

Figure 1.11 Possible role played by IL-6 in the shift from acute to chronic inflammation. Stage 1: following acute inflammatory response, IL-6 can bind with sIL-6R. Stage 2: trans-signaling through gp130 leads to monocyte recruitment. Stage 3: prolonged IL-6 leads to neutrophilic apoptosis, phagocytosis and mononuclear accumulation at the site of injury. IL, interleukin; JAK, Janus activated kinase; MCP, monocyte chemoattractant protein; PMN, polymorphonuclear neutrophil; sIL-6R, soluble IL-6 receptor (Gabay, 2006).

IL-6 also plays a role in leukocyte recruitment in vivo. A complex of IL-6 and IL-6Rα elicits endothelial cells to secrete IL-8 and monocyte chemoattractant protein-1 (MCP-1) and also induces expression of adhesion molecules (Romano et al., 1997). Expression of IL-6Rα is limited to leukocyte and hepatocyte membranes, but it can shed from the neutrophil membrane as a soluble form and this form is found in high concentrations in neutrophil enriched inflammatory fluids (Romano et al., 1997; Hurst et al., 2001). The sIL-6Rα then combines with IL-6 to bind
gp130 on the membranes of stromal cells in a mechanism termed as trans-signaling (fig. 1.11).

IL-6 favours the transition from neutrophil to monocytes in inflammation (Hurst et al., 2001). Neutrophils stimulated with inflammatory cytokines can selectively produce MCP-1 which facilitates late monocyte recruitment (Yamashiro et al., 1999). Endothelial activation by pro-inflammatory molecules leads to secretion of chemoattractants like platelet activating factor, IL-8 and IL-6 which recruits neutrophils and induce IL-6Ra shedding from their membranes. The combination of IL-6Ra with IL-6 enables ligation to gp130 on the endothelial cell membrane and increases IL-6 and MCP-1 secretion favouring transition from neutrophil to monocytes recruitment (Gabay, 2006) (fig. 1.5).

1.4 Cell signaling cascades in inflammation

1.4.1 NF-κB activation and inflammatory response

NF-κB is a master regulator of immune response and an ancient signaling pathway in host defense of multicellular organisms (Karin et al., 2005). NF-κB comprises of a family of inducible transcription factors that serve as important regulators of host immune and inflammatory response. The family includes NF-κB1 (p50/p50), NF-κB2 (p52/p100), p65 (RelA), RelB and cRel (Chen et al., 1999). These proteins share a 300 amino acid domain designated the Rel homology domain which mediates DNA binding dimerization and nuclear transport of NF-κB proteins (Baldwin, 1996; Ghosh et al., 1998). Most members of this family except RelB can homodimerize as well as heterodimerize. The most prevalent activated form of NF-κB is a heterodimer consisting of p50 or p52 subunit and p65 which contains transactivation domains necessary for gene induction (Yamamoto and Gaynor, 2001).

NF-κB regulates host inflammatory immune responses and cellular growth properties by increasing the expression of specific cellular genes (Baldwin, 1996; Barkett and Gilmore, 1999; Chen et al., 1999). These include genes encoding cytokines, chemokines, receptors involved in immune recognition such as members of MHC, proteins involved in antigen presentation and receptors required
for neutrophil adhesion and migration (Pahl, 1999). Cytokines also stimulate NF-κB. Cytokines such as IL-1β and TNF-α directly activate NF-κB pathway and establish a positive autoregulatory loop that amplify the inflammatory response and increase the duration of chronic inflammation (Pahl, 1999; Yamamoto and Gaynor, 2001).

NF-κB also stimulates the expression of enzymes like iNOS and COX-2 (Pahl, 1999; Connelly et al., 2001). The NF-κB pathway is likewise important in the control of the immune response. It modulates B-lymphocyte survival, mitogen dependent cell proliferation and isotype switching leading to the differentiation of B-lymphocytes into plasma cells (Gerondakis et al., 1998). In addition, NF-κB also regulates IL-2 production and increases the proliferation and differentiation of T lymphocytes (Gerondakis et al., 1998; Pahl, 1999).

**Regulation of NF-κB pathway**

NF-κB system is a cytoplasmic sensor that responds to immune assaults and variety of external and internal danger signals like oxidative stress, hypoxia and genotoxic stress (Karin et al., 2005). In unstimulated cells, the NF-κB proteins are localized in the cytoplasm, associated with a family of inhibitor proteins known as IκB (IκBa, IκBβ, IκBe) (Baldwin, 1996; Ghosh et al., 1998). The IκB proteins contain several distinct domains like:

- ankyrin repeats that are critical for IκB interactions with NF-κB
- an NH₂-terminal regulatory domain that is a target for the inducible phosphorylation and subsequent ubiquitination of IκB
- COOH-terminal PEST domain that is important in regulating IκB turnover.

The IκB proteins bind to NF-κB and block their nuclear translocation signal. A variety of stimuli including cytokines such as TNF-α and IL-1, phorbol esters, lipopolysaccharide (LPS), viral infection, the human T-cell leukemia virus type-1 transforming protein Tax, ultraviolet radiation and free radicals result in the nuclear translocation of NF-κB (Pahl, 1999).

The phosphorylation of IκB proteins mediated by IκB kinase (IKKs) is a key step involved in the regulation of Rel / NF-κB complexes. IκB kinase consists
of IKK-α and IKK-β and the associated immunomodulatory protein IKK-γ or NEMO (NF-κB essential modulator) (Zandi and Karin, 1999). The activated IKK complex phosphorylates the IKB proteins on two closely spaced serine residues in the amino terminus of these proteins (Baldwin, 1996; Ghosh et al., 1998). Phosphorylation of IKB leads to its ubiquitination on two amino-terminal lysine residues by the E3-ubiquitin ligase complex, thus targeting it for degradation by the 26S proteosome. Freed from association with IKB subunits, the NF-κB proteins translocate to the nucleus where they bind to specific elements in the promoter regions of target genes to activate gene expression (Zandi and Karin, 1999).

1.4.2 Mitogen activated pathway kinases (MAPKs) and inflammation

The other major extracellular signal transduction pathway stimulated by inflammatory mediators is the mitogen activated protein kinase (MAPK) pathway (Guha and Mackman, 2001). MAPKs are a family of serine/threonine protein kinases composed of p44 and p42 isoforms (also known as extracellular signal receptor activated kinase ERK-1 and ERK2), p38 and c-Jun NH₂-terminal kinase (JNK). MAPK cascades consist of a module that includes three kinases. These establish a sequential activation pathway comprising of a MAPK kinase kinase (MKKK), a MAPK kinase (MKK) and MAP kinase (fig. 1.12).

MKKKs are serine/threonine protein kinases that phosphorylate and activate MKKs, while MKKs are dual specificity protein kinases that phosphorylate the threonine and tyrosine residues of a conserved T-X-Y motif of activation loop of MAPKs. The X residue is different in each class of MAPKs: ERK has a threonine-proline-tyrosine (T-P-Y) motif, JNK has a threonine-proline-tyrosine (T-P-Y) motif and p38 has a threonine-glycine-tyrosine (T-G-Y) motif. Phosphorylation of T-X-Y motif activates the MAPKs allowing phosphorylation of a specific repertoire of cytoplasmic and nuclear proteins including various transcription factors. The substrate specificity is influenced by the X residue of the T-X-Y motif and by the amino acids surrounding this motif (Dong et al., 2002; Johnson and Lapadat, 2002).

MAPKs have predominant role in mediating inflammation and arthritis (Thalhamer et al., 2008). It has been found that LPS, the key mediator in the
inflammation response, can induce activation of these MAPK proteins in mononuclear cells and other cell types (Guha and Mackman, 2001). Studies have shown that p38, ERK and JNK play role in induction of various inflammatory mediators like cytokines and chemokines (Rawadi et al., 1998; Carter et al., 1999).

![Diagram of MAPK cascades](image)

**Figure 1.12 The ERK, JNK and p38 MAPK cascades.** There are three major classes of MAPKs in mammals, the ERKs and the two stress activated protein kinase (SAPK) families: JNK and p38. MAPKs are activated via a signaling cascade that is conserved from yeast to mammals in which stimulation of MAPKs requires the upstream activation of a MAPK kinase (termed MAPKK, MEK or M KK) and a MAPK kinase kinase (termed MAPKKK, MEKK or MKKK). Activation of the MAPKs results in phosphorylation of a specific repertoire of cytoplasmic and nuclear target proteins including various transcription factors. Negative feedback mechanisms including MAPK phosphatases (MKPs) exist to ensure MAPK enzymes are not activated constitutively. ASK, apoptosis signal-regulating kinase; ATF, activating transcription factor; ERK, extra-cellular signal-regulated kinase MAPK; JNK, c-Jun NH2-terminal kinase MAPK; MEK, MAPK kinase; MEKK, MEK kinase; MLK, mixed lineage kinase; MKK, MAPK interacting serine/threonine kinase; MAPKAP, MAPK activated protein kinase; cPLA2, cytosolic phospholipase A2; MYC, c-Jun NH2-terminal kinase MAPK kinase; Elk, Ets-like transcription factor; Ets, E26-AMV virus oncogene cellular homologue; TAK, TGF-β-activated kinase; PAC, phosphatase of activated cells; MKP, MAPK phosphatase.
Introduction

Riedemann et al. (2003) showed that LPS-induced IL-6 production was dependent on p38 and ERK 1/2 activation (phosphorylation) but not on engagement of JNK1/2. In tubular epithelial cells of the kidney, LPS-induced generation of IL-6 was dependent on activation of both p38 MAPK and ERK (Leonard et al., 1999b). Cytokines like TNF-α, IL-1 and IL-6 were reported to activate all three MAPKs in synovial fibroblasts with consequent induction of MMP-1 and MMP-13 (Liacini et al., 2003). Activation of p38 by LPS resulted in the stimulation of NF-κB specific DNA protein and the subsequent expression of iNOS and NO release (Chiang et al., 2005). These data point out the essential role of MAPKs in mediating an inflammatory response.

Regulation of MAPKs

Many different receptor types are able to activate MAPK cascades by multiple mechanisms including tyrosine kinase receptors, G protein coupled receptors and cytokine receptors (English et al., 1999; Widmann et al., 1999). Negative feedback mechanisms exist to ensure that MAPK enzymes are not activated constitutively. MAPKs induces three different types of protein phosphatases that dephosphorylate and inhibit MAPK: dual specificity phosphatases (DUSPs), threonine phosphatases and tyrosine phosphatases (Camps et al., 2000; Jeffrey et al., 2007).

Abnormalities associated with inflammation comprise a large, officially unrelated group of disorders which underlie a vast variety of human diseases. The immune system is often involved with inflammatory disorders and many immune system disorders result in abnormal inflammation. Non-immune diseases with an etiological origin in inflammatory processes include cancer, atherosclerosis, and ischemic heart disease (Cotran et al., 1998). Some of the disorders associated with inflammation include asthma, chronic prostatitis, glomerulonephritis, inflammatory bowel disease, cancer, transplant rejection, sepsis, arthritis etc.
1.6 Arthritis

Arthritis simply means ‘inflammation of the joints’. The word ‘rheumatism’ is even more general, and is used to describe aches and pains in joints, bones and muscles. There are more than 200 types of arthritis and rheumatic disease. The general symptoms of arthritis include pain, swelling and stiffness especially morning stiffness with limitation of joint movement (Tenny et al., 2004). ‘October 12th’ is considered ‘World Arthritis Day’. The World Arthritis Day has gained importance because this pervasive disease has affected more than 1% of the human population and is a leading reason for reduced life expectancy (Firestein et al., 2001). The consequent morbidity and mortality has a substantial socio-economic impact and epidemiology of arthritis in female : male is 3:1. Majority of patients with a more aggressive disease evolution become clinically disabled within 20 years (Narendhirakannan et al., 2007).

1.7 Architecture of articular cartilage

The normal articular joint capsule consists of thick fibrous portion lined by a thin subsynovium (lamina propria) and the synovium, which is in contact with the synovial fluid. Synovium contains synoviocytes, a continuum of cells that have both secretory and phagocytic functions. Type A synoviocytes are synovial lining cells that can phagocytose and pinocytose. Type B synovial lining cells synthesize hyaluronan which is secreted into synovial fluid at the plasma membrane, as well as the other components of the extracellular matrix of the synovium. Lubricin, a glycoprotein involved in the boundary lubrication of cartilage is also synthesized by the synovium (Mcilwraith, 1996).

Articular cartilage is translucent and has a glass-like (hyaline) appearance, that is due primarily to its high water content (70% - 80% by weight) and the very fine structure of its collagen (most of it type II) fibril network. It is composed by chondrocytes and an extracellular matrix. In addition to collagen, that forms a 3-dimensional meshwork (Mayne, 1989), the cartilage also contains proteoglycans like hyaluronan and aggrecan (Mcilwraith, 2002). The articular cartilage is subdivided into three unmineralized zones. In adult animals, zone I or superficial zone
(fig. 1.13) has the highest cell density. The chondrocytes are relatively small and flat, and oriented with the long axes parallel to the surface. Zone II (transitional zone) shows larger and rounded cellular profiles. In zone III (radiate zone), the cells are larger and arranged with their long axes perpendicular to the surface (Schenk et al., 1986). Thirteen types of collagen have been isolated from the articular cartilage matrix including types II, V, VI, IX, X and XI collagen (Mayne, 1989). The function of type II collagen is to provide tensile stiffness to the cartilage.

The widely dispersed and heterogeneous population of proteoglycans and glycosaminoglycan present within the articular cartilage ground substance provides compressive stiffness to the articular cartilage. The presence of highly charged, polyanionic sulphate and carboxyl groups on the glycosaminoglycans, chondroitin sulphate and keratan sulphate, combined with the high molecular weight of the proteoglycan aggrecan, is responsible for the large osmotic pressure created within the articular cartilage. The effect of the glycosaminoglycan is to draw water into the tissue and to expand the collagen matrix. The resistance of the tightly packed collagen fibrils to this expansion provides the articular cartilage with a great
capacity to resist compressive forces (Greenwald et al., 1978). Any alterations in
the structure or quantity of the glycosaminoglycan will result in a change of
compressive stiffness of the articulate cartilage (Sledge, 1993).

1.8 Osteoarthritis and Rheumatoid arthritis

The common types of arthritis are:

- Osteoarthritis
- Rheumatoid arthritis

Osteoarthritis (OA), the most prevalent disorder of the musculoskeletal
system, is believed to be a consequence of mechanical and biological events that
destabilize the normal coupling of degradation and synthesis within articular joint
tissues. In OA knees, pathogenic changes result in cartilage erosion, meniscal
degenerative tears, subchondral bone remodeling, osteophyte formation and
synovial inflammation. The degradation of OA cartilage matrix has been shown to
be related to the excess synthesis of a large number of matrix metalloproteinases
like gelatinase-A (MMP-2) and gelatinase-B (MMP-9) which increases the
pathologic chondral, meniscal and synovial lesions of OA (Hsu et al., 2004).
Interleukin-1β and tumor necrosis factor-α are predominant pro-inflammatory
cytokines synthesized during the OA. These are found in elevated levels in the
synovial membrane, the synovial fluid and the cartilage of OA patients and play an
essential role in the cartilage destruction and inflammation process (Fernandes
et al., 2002).

Rheumatoid arthritis (RA) is a chronic inflammatory disease affecting
predominantly lining structures, including synovial tissue, tendon sheaths, bursae,
pleura and pericardium. (Feldmann et al., 1996). The course of RA is
characterized by alternating remissions and exacerbations and long term treatment
is required to prevent disease flares (Kvien, 2004). It usually affects people
between 25 years and 55 years of age at initial onset. It can affect any joint, but is
more common in peripheral joints, such as the hands, fingers and toes. RA can
cause functional disability, significant pain, cartilage and joint destruction, leading
to deformity and premature mortality.
1.9 **Pathogenesis of rheumatoid arthritis**

An autoimmune-mediated attack by autoantibodies like rheumatoid factor (RF) or anticyclic citrullinated peptide (CCP) antibody on the joint is the main reason for the disease pathology (Jawaheer et al., 1994). Nielen and colleagues (2004) examined the relationship between these autoantibodies and eventual diagnosis of RA patients and found that RF and anti-CCP antibody were not present in every patient and likely to be found in patients with more severe symptoms. Other studies reported that an unidentified trigger stimulates the production of autoantibodies by B lymphocytes several years before the eventual onset of clinically evident RA (Rantappa-Dahlqvist et al., 2003; Berglin et al., 2004).

T lymphocytes are cells central to the pathogenesis of RA (Firestein, 2003). The importance of T cells in the pathogenesis of RA is supported by the fact that T cells largely accumulate in the synovium of RA patients and that these cells are capable of transferring disease to immunodeficient mice (Mima et al., 1995). It was also observed that pathologic T cell depletion prevents collagen induced arthritis in mice (Haqqi et al., 1996). CD4+ T cells initiate and regulate several cell mediated immune processes that cause synovial inflammation and joint destruction (fig. 1.14). Activated T cells release chemical mediators like interferon-γ and interleukin-17, which stimulate the activity of other immune cells like B-cells, monocytes, macrophages and fibroblasts. These stimulated immune cells then release chemical mediators like inflammatory cytokines IL-1, IL-6, TNF-α as well as prostaglandins and nitric oxide that induce inflammation and joint damage (Huet et al., 1993; Arend and Dayer, 1995; Firestein, 2003). These pro-inflammatory mediators shift the whole body protein metabolism towards net catabolism, elevates resting energy expenditure and increases joint pain and stiffness (Rall and Roubenoff, 2004).

Kaneko et al. (2001) reported co-localization of cathepsins, MMPs like collagenase-1, collagenase-3, stromelysin-1, gelatinase-A and gelatinase B and inflammatory cytokines at the sub-chondral region of RA patients. These tissue damaging enzymes cause proliferation of synoviocytes and formation of
fibroblastic type synovial lesions which along with angiogenesis leads to the formation of a very aggressive tissue called ‘pannus’ (Jawaheer et al., 1994; Feldmann et al., 1996; Koch, 1998). Expansion of the pannus induces bone erosion and cartilage thinning, leading to the loss of joint function. One of the earliest phenomena observed in RA is synovial neovascular formation delivering nutrients and oxygen to this proliferating pannus (Folkman, 1995). The rheumatoid pannus can thus be considered a local tumour.

Figure 1.14 T lymphocytes in the pathogenesis of rheumatoid arthritis. Antigen bearing dendritic cells (DC) in the lymph node activates T cells to proliferate and differentiate. Activated T cells are essential for the initiation of the immunologic cascade underlying rheumatoid arthritis like activation of B cells, macrophages (MΦ) and fibroblast like synoviocytes (FLS).

Another mediator which plays a pivotal role in the pathogenesis of RA is vascular endothelial growth factor (VEGF) (Koch et al., 1994). The VEGF level in synovial fluid and tissues correlates with the clinical severity of RA and with the degree of joint destruction (Hitchon et al., 2002). VEGF mediates the recruitment, chemotaxis and proliferation of osteoclast precursor macrophages, leading to bone destruction (Henriksen et al., 2003). Thus combined activities of many such pro-inflammatory mediators ultimately result in bone erosion, cartilage destruction and restriction of joint movement (fig. 1.15).
1.10 Risk factors of rheumatoid arthritis

The likelihood of developing arthritis is influenced to a certain degree by genetic factors. Studies have examined that the concordance rates for RA were higher in monozygotic twins (12-15%) than in dizygotic twins (3-4%) (Silman et al., 1993). Polymorphisms of several genes that are important in the regulation of immune response have been linked to an increased risk of developing RA. Many high-risk alleles like HLA-DQB1, HLA-DQA1 and HLA-DRB1 have been identified in MHC class II genes. The HLA-DR4 region of the HLA-DRB1 cluster is strongly associated with an increased risk of severe and persistent RA (Zanelli et al., 2000). Wordworth (1989) reported that majority of patients with RA possessed the DRB*0101, DRB*0401, DRB*0404 and DRB*0405 genetic allotypes. Individuals with these allotypes have a four fold greater risk of RA development compared to that of the general population. Moreno et al. (1996) reported that combined expression of susceptibility alleles can lead to greater risk and increased severity of RA. RA is initiated when RA associated MHC molecules present self-
Introduction

antigens to autoreactive cells (Zanelli et al., 2000). Epidemiological studies conducted showed that the prevalence of RA is considerably higher (5-8%) in populations like Chippewa and Pima American tribes (Kvien, 2004). Studies have also shown that somatic mutation in the p53 gene of rheumatoid synoviocytes is a contributory factor of RA (Firestein et al., 1997; Han et al., 1999).

Women are more predisposed to RA than men. Severe clinical disease activity, structural damage and deformities have been reported equally in both genders in RA. Generally, however, women report more severe symptoms and greater disability and often have higher work disability rates compared with men (Abdel-Nasser et al., 1997). As in the general population, men with RA have considerably higher mortality rates than women (Sokka et al., 2009). In women somehow, pregnancy ameliorates RA. Pregnancy is characterized by decreased production of T-helper cell Th\(^1\) associated cytokines like IL-1 and interferon-gamma and increased production of Th\(^2\) associated cytokines like IL-4 and IL-10, as well as decreased production of pro-inflammatory cytokines like TNF-\(\alpha\) and IL-12 (Munoz-Valle et al., 2003). This altered cytokine profile may be one of the mechanisms responsible for the ameliorating effects of pregnancy on RA.

Studies have revealed that blood transfusion is another risk factor for RA. Blood transfusions can induce rheumatoid factor production (Celada et al., 1984) and overall immune suppression in people receiving transfusions (Bordon et al., 1995). In addition, differences between HLA antigens on the donor and host white blood corpuscles may lead to the development of alloantibodies and down regulation of specific T-cell clones (Van der Mast et al., 1994).

Many viruses like Epstein Barr virus, Pavo virus, Rubella virus and bacterial organisms like mycoplasma, mycobacterium and Yersinia are reported to trigger arthritis by infecting host cell and altering the reactivity of responsiveness of T cells (Firestein, 2001). However no agents have been conclusively shown to be causative. Other factors contributing to RA are obesity (Symmons et al., 1997), cigarette smoking (Voigt et al., 1994) and certain medications like interferon-\(\alpha\) (which is used to cure hepatitis) (Harrison et al., 1997).
1.11 Complications associated with rheumatoid arthritis

RA is a major cause of disability. It was reported that half of people diagnosed with arthritis stop work within ten years (Koopman, 2001). People with rheumatoid arthritis also have a greater risk of lymphoma. A study that included 11,000 arthritic patients in Sweden reported that these patients were at a higher risk of developing prostate and lung cancers (Baecklund et al., 1998). Rheumatoid catexia is another problem associated with RA. Rheumatoid catexia leads to muscle weakness, osteoporosis, loss of functional capacity and increased susceptibility to infection which accelerates morbidity and mortality in arthritic patients (Orwoll et al., 2000; Rall and Roubenoff, 2004).

Patients with RA do not have decreased fertility. Most studies showed an increase in nulliparity, whereas parity rates in fertile RA women were unchanged. However, they may require a prolonged time to conceive. Decreased sexual drive, pain, ovulation dysfunction and an impaired hypothalamic-pituitary-adrenal axis may be responsible for these findings (Cunningham et al., 2003).

1.12 Inflammatory mediators involved in the progression of rheumatoid arthritis

1.12.1 Role of lipid mediators in the progression of rheumatoid arthritis

Lipid mediators have profound role in RA progression. Synovial tissues of patients with RA have increased expression of COX-2 (Crofford, 1994). COX-2 expression in most tissues is highly regulated with rapid induction in response to inflammatory stimuli. Increased COX-2 expression in synovial tissues is driven by the pro-inflammatory cytokines like IL-1 and TNF-α (Crofford et al., 1994). These cytokines stimulate COX-2 transcription by activating transcription factors including NF-κB and c/EBP (Crofford et al., 1994). In addition to cytokine networks, signaling via cell surface integrins also increases the expression of COX-2 (Crofford, 1999).

Evidence provided by animal models of inflammatory arthritis strongly suggested that increased expression of COX-2 was responsible for increased PG production seen in inflamed joint tissues (Anderson et al., 1996; Portanova et al.,
Introduction

Anderson et al. (1996) reported that in rat adjuvant arthritis, marked edema of hind footpad coincided with local PGE$_2$ generation, which associated with upregulation of COX-2 mRNA and protein in the affected paws. Kang et al. (1996) reported that synovial cells of RA patients exhibit extensive and intensive intracellular COX expression. Seki et al. (2004) reported increased COX-2 expression \textit{in vivo} by specific immunohistochemical analysis in the synovial tissue with internal derangement. This may be involved in angiogenesis of the synovial membrane and erosion of cartilage in inflamed joints (Myers et al., 2000; Woods et al., 2003). Crofford et al. (1994) demonstrated that IL-1 enhanced \textit{de novo} synthesis of COX-2 mRNA and protein in rheumatoid synovial explants and synoviocytes. Anderson et al. (1996) reported that increase in PGE$_2$, COX-2 mRNA and protein levels in the paw tissue of adjuvant induced rats can be suppressed and returned to baseline values on treatment with SC-58125, a selective COX-2 inhibitor. Therapeutic administration of anti-PGE$_2$ monoclonal antibody 2B5 was observed to decrease COX-2 levels \textit{in vivo} (Portanova et al., 1996).

It was previously shown that LOX pathways are also important in the RA inflammatory process and that synovial fluid and serum from RA patients contains high amounts of leukotrienes (Klickstein et al., 1980; Gursel et al., 1997). Chen et al. (2006) reported that neutrophil derived leukotrienes B$_4$ was required for arthritis development. Experiments using 5-lipoxygenase-activating protein (FLAP) deficient mice (Griffiths et al., 1997) and 5-LOX deficient mice (Chen et al., 1994) confirmed that leukotrienes played essential role in experimentally induced arthritis. Although macrophages and mast cells, produce substantial amounts of LTB$_4$ (Lewis et al., 1990), neutrophils are the main producers of LTB$_4$ in rheumatoid joints (Elmgreen et al., 1987) and these cells initiate destruction in the joints by secreting proteases and reactive oxygen species and promoting synthesis of matrix metalloproteinases (Ahluwalia et al., 2007). A critical contribution of neutrophil-derived LTB$_4$ to arthritis induction and severity has been revealed in a mouse serum transfer model of inflammatory arthritis (Chen et al., 2006). In this study it was shown that mice lacking 5-LOX or leukotriene A$_4$ hydrolase enzymes were protected from developing the disease and that there was a specific requirement for LTB$_4$ and not other leukotrienes for the pathogenesis in
Introduction

this model. Koshihara et al. (1988) and Ahmadzadeh et al. (1991) reported that synovial fluid levels of LTB₄ and sulfidopeptide leukotrienes were substantially higher in patients with rheumatoid arthritis and the synovial fluid levels of leukocytes, immune complexes and rheumatoid factor directly correlated with LTB₄ levels in these patients (Ahmadzadeh et al., 1991).

Studies in animal models (acute inflammation and arthritis model) with different leukotriene inhibitors (inhibitors of leukotriene synthesis or leukotriene antagonists) showed that LTB₄ inhibitors had a clear effect on immune complex-induced models and collagen-induced arthritis (Griffiths et al., 1995). Clinical trials with LTB₄ inhibitors in chronic inflammatory arthritis were limited (Weinblatt et al., 1992); however, the effect of methotrexate, the reference treatment in RA, was reported to be partly due to LTB₄ synthesis inhibition (Sperling et al., 1992). Recently Gheorghe et al. (2009) reported the modulatory effect of intraarticular administration of glucocorticoids on 5-LOX and 15-LOX expression in synovial tissue. Intraarticular administration of glucocorticoids decreased 5-LOX expression whereas its effect on 15-LOX was less evident. These data shed light to the fact that modulation of LOX during arthritis can alleviate disease progression (Sperling et al., 1992).

1.12.2 Role of nitric oxide in progression of rheumatoid arthritis

Several lines of evidence have indicated nitric oxide (NO) as contributing factor in the pathogenesis of arthritis (Clancy et al., 1998) (fig. 1.16). High concentrations of nitrites and nitrates were found in the synovial fluid and plasma of patients with RA (Farrell et al., 1992). Activated chondrocytes, fibroblasts, synoviocytes and macrophages in synovial fluid and peripheral blood mononuclear cells of RA patients exhibited increased expression of iNOS and produced excess NO that correlated well with disease activity (St Clair et al., 1996; Jang and Murrel, 1998; Migita et al., 2002; Lotito et al., 2004). Studies using experimental animal models suggested that excessive levels of NO increased synovial blood flow and modulated cellular function within synovium and articular cartilage (Cuzzocrea et al., 2000). Bezerra and coworkers (2004) examined the effects of NO and peroxynitrite in zymosan induced arthritis and found that addition of
peroxynitrite scavenger uric acid ameliorated synovitis and glycosaminoglycan loss.

Cytokines have prominent role in the expression of iNOS (Taskiran et al., 1994; Clancy et al., 1998). Van’t Hof and Ralson (2001) reported that in osteocytes, IL-1 and TNF-α caused activation of iNOS pathway and NO release which potentiated bone loss leading to osteoporosis in RA. Within the joint, NO produced in response to cytokine stimulation inhibits proteoglycan and collagen synthesis, activates MMPs, increases susceptibility to oxidants, decreases expression of IL-1 receptor antagonist, inhibits actin polymerization and apoptosis causing degradation of articular cartilage, (Taskiran et al., 1994; Murrell et al., 1995; Pelletier et al., 1996; Clancy et al., 1998) (fig. 1.15).

Reports on the analysis of NO content in the synovial fluid of patients with arthritis yielded contradictory findings (Haklar et al., 2002). Experimental
evidence suggested that NO and its derivatives can play a protective role like enhancing collagen synthesis (Xia et al., 2006). When cells harvested from the torn edges of tendons from patients (who underwent rotating cuff surgery) were transfected with an adenovirus containing gene for iNOS or treated with S-nitroso-N-acetylpencillamine (SNAP), total protein and collagen synthesis were enhanced (Xia et al., 2006). Hancock and Riegger-Krugh (2008) demonstrated that small amounts of transiently produced NO, produced by eNOS decreased pain associated with arthritis. Thus the interactions between the iNOS and COX pathways have led to controversial results depending on the cell system and experimental conditions.

*In vitro* it was reported that NO attenuated synthesis of PGE$_2$ in chondrocytes from arthritic patients (Amin et al., 1997). There was a report stating that NO potentiated cytokine induced PGE$_2$ production in vascular smooth muscle cells, human microglial cells and human airway epithelial cells (Rojas et al., 2003). Conversely, in a murine air pouch model of granulomatous inflammation, NO produced by iNOS inhibited induction of COX-2 (Vane et al., 1994).

Clancy et al. (2004) reported that NO and peroxynitrite opposed each others effects on NF-κB activation in chondrocyte cultures. When bovine chondrocytes were incubated with IL-1β, 40% of the cells showed positive immunostaining for NF-κB subunit in the nucleus which was decreased by addition of NO donor S-nitrocysteine ethyl ester. However, incubation with IL-1β and peroxynitrite resulted in an increase in cells with activated NF-κB from 40% to 73% illustrating opposing effects of NO and ROS. These data indicate the importance of NF-κB in NO activation.

1.12.3 *Role of cytokines in the progression of RA*

Several reports have stated the role of cytokines in the progression of RA (Miossec et al., 1990; Choy and Panayi, 2001). During arthritis, activated T cells stimulate monocytes and synovial cells in synovium to produce significant amounts of metalloproteinases and pro-inflammatory cytokines like IL-1, GM-CSF, TNF-α and IL-6 which initiates destruction of cartilage and underlying bone (Firestein and Zvaifler, 1990; Miossec et al., 1990). Two major mediators that play role during arthritis are TNF-α and IL-6.
TNF-α plays a significant role in the progression of RA (Van den Berg, 2000). TNF-α is released primarily by monocytes, macrophages and is also released by B cells, fibroblasts and T cells. TNF-α produces direct inflammatory effects by increasing the expression of cell-surface adhesion molecules for migration of leukocytes to the inflammatory tissue (Choi and Panayi, 2001). It promotes inflammation by stimulating the release of other pro-inflammatory cytokines like IL-1, IL-6 and IL-8. TNF-α induces the production of MMPs, reactive oxygen species and nitric oxide during arthritis by synoviocytes or chondrocytes (Tracey and Cerami, 1990). TNF-α also stimulates the production of prostaglandin E\textsubscript{2} and collagenase in synovial cells and human articular chondrocytes in vitro (Dayer et al., 1985; Bunning RA, Russell, 1989). Deleuran et al. (1992) reported co-expression of TNF-α and both its cell-associated receptors, p55 TNFR (Type I) and p75 TNFR (Type II) in synovial tissue sections of arthritic patients. Van den Berg (2000) demonstrated that arthritis progression was strongly reduced in TNF knock-out mice.

Another cytokine that has a profound role during arthritis is IL-6 (Sasai, 1999). The major sources of IL-6 are synovial fibroblasts, activated macrophages and lymphocytes (Yoshizaki et al., 1998). Alonzi et al. (1998) reported that high amount of IL-6 is required for the development of arthritis. Increased production of IL-6 leads to common clinical manifestations like weight loss, fatigue and poor appetite (Yoshizaki et al., 1998). Madhok et al. (1993) reported a positive correlation between elevated levels of IL-6 and disease activity parameters like duration of morning sickness and articular index. Dasgupta et al. (1992) identified an association between serum IL-6 levels and the severity of bone destruction. Excess production of IL-6 also contributes to anemia in RA patients by increasing hepcidin production and enhancing thrombocytosis through increased megakaryocyte differentiation (Andrews, 2004).

IL-6 also induces B-cell differentiation, hypergammaglobulinemia and production of autoantibodies like rheumatoid factor and autoantibodies to citrullinated peptides (Yoshizaki et al., 1998). IL-6 initiates proliferation of synovial fibroblasts, osteoclast activation and differentiation, recruitment of
mesenchymal vascular cells, neoangiogenesis, leukocyte accumulation and damage to cartilage during arthritis (Tamura et al., 1993; Giraudo et al., 1996; Romano et al., 1997; Kudo et al., 2003; Mihara and Norihiro, 2005; Park and Pillinger, 2007).

Atreya et al. (2000) stated that IL-6 favoured mononuclear cell accumulation at the site of injury, through continuous MCP-1 secretion, angioproliferation and anti-apoptotic functions on T cells. IL-6 induces endothelial expression of adhesion molecules and endothelial production of monocyte chemoattractant protein-1, a chemokine that is responsible for monocyte recruitment in joints (Gabay et al., 2006; Romano et al., 1997).

A few controversial reports have demonstrated that IL-6 lacks many typical pro-inflammatory properties and furthermore exerts a number of anti-inflammatory activities. IL-6 does not directly stimulate the production of collagenase, matrix metalloproteinase or stromelysin (Akira et al., 1993), although it does potentiate IL-1 and TNF-stimulated collagenase and prostaglandin E\textsubscript{2} production by chondrocytes (Van de Loo et al., 1997). IL-6 is a potent inducer of TIMP-1 (Shingu et al., 1995). In a model of arthritis, IL-6 significantly enhanced synthesis of TIMP-1 in chondrocytes, inhibited superoxide production and suppressed spontaneous and IL-1-mediated degradation of cartilage matrix (Shingu et al., 1995).

**1.12.4 Role of NF-κB in rheumatoid arthritis**

Studies conducted on animal models of inflammatory arthritis showed that NF-κB activation plays a pathogenic role *in vivo*. Tsao (1997) reported that synovial NF-κB activation occurred few days after immunization in rat adjuvant arthritis. Tak (2001) demonstrated that intra-articular transfer of a functional IKK-β gene in normal rats selectively activated NF-κB and rats then exhibited clinical signs of arthritis. Conversely, reduction of NF-κB nuclear translocation and clinical synovitis occurred in rats after an intra-articular delivery of dominant-negative adenoviral IKK-β construct (Tak, 2001).

**1.12.5 Oxidative stress and rheumatoid arthritis**

Several lines of evidence have suggested that free radical mediated oxidative stress play role in the pathogenesis of RA (Rowley et al., 1984; Dai et al.,
A free radical can be defined as a chemical species, an atom or a molecule that has one or more unpaired electrons in its valence shell and is capable of existing independently. Most common radicals are derivatives of oxygen like superoxide free radical anion (O$_2^-$), hydroxyl free radical (OH$^-$), lipid peroxyl (LO$^-$), lipid alkoxyl (LOO$^-$), lipid peroxide (LOOH) as well as non-radical derivatives such as hydrogen peroxide (H$_2$O$_2$) and singlet oxygen.

Free radicals / reactive oxygen species are produced mainly from cellular metabolism like mitochondrial electron transport chain, endoplasmic reticulum oxidation, NADPH oxidase, xanthine oxidase, prostaglandin synthesis, reduced riboflavin, nitric oxide synthase, reperfusion injury, cytochrome P$_{450}$, activated neutrophils and phagocytic cells and environmental sources like drugs, pesticides, transition metals, tobacco smoke, alcohol, radiations and high temperature (Mahajan and Tandon, 2004). Normally, equilibrium exists between free radical / reactive oxygen species formation and endogenous antioxidant defense mechanisms but if this balance is disturbed, oxidative stress results. This state of oxidative stress finally results in injury to tissues like cartilage (Panasyuk et al., 1994; Rees et al., 2003; Rees et al., 2004).

Analysis of tissue samples and synovial fluid of arthritic patients showed the presence of lipid peroxidation products in these samples indicating oxidative stress (Rowley et al., 1984; Dai et al., 2000; Taysi et al., 2002; Dalle-Donne et al., 2003). Heliovaara et al. (1994) and Henrotin et al. (2003) reported that oxygen and nitrogen radicals generated during oxidative stress damage extracellular matrix and cellular elements in cartilage by upregulating mediators of matrix degradation, inhibiting synthesis of matrix components like proteoglycans type II collagen as well as sulfation of newly synthesized glycosaminoglycans.

Many reports suggested that oxygen radicals fragment hyaluronic acid and chondroitin sulfate and damage hyaluronan binding region of the proteoglycan core protein, thereby interfering with proteoglycan-hyaluronan interactions (Panasyuk et al., 1994; Rees et al., 2003; Rees et al., 2004). Oxygen radicals also induce T cell hyporesponsiveness in RA patients through its effects on proteins and...
proteosomal degradation (Lotz et al., 2003). Another major feature is that oxygen radicals initiate oxidation of local LDL that promotes upregulation of neighboring adhesion molecules and chemokines and accelerates the process of atherosclerosis in RA patients (Winyard et al., 1993; Dai et al., 2000).

Studies have demonstrated that increased lipid peroxidation along with decreased antioxidant status accelerates arthritis development (De Leo et al., 2002, Taysi et al., 2002; Vijayakumar et al., 2006). Karatas et al. (2003) reported raised levels of malondialdehyde and low levels of endogenous antioxidants in patients of rheumatoid arthritis. Plasma catalase activity was reported lower in patients with RA by Kamanli et al., 2004. Another study reported impaired glutathione reductase activity in synovial fluid of RA patients (Bazzichi et al., 2002).

Epidemiologic studies have shown that an inverse association exists between dietary intake of antioxidants and RA incidence (Heliovaara et al., 1994; Mulherin et al., 1996; Paredes et al., 2002; Bae et al., 2003; Cerhan et al., 2003; Hagfors et al., 2003). Vitamin E uncoupled joint inflammation and joint destruction in the transgenic KRN / NOD mouse model of RA, with a beneficial effect on joint destruction (Bandt et al., 2002). Another study suggested that therapeutic co-administration of antioxidants along with conventional drugs to RA patients resulted in statistically significant increase in the concentration of endogenous antioxidants, decrease in the concentration of MDA along with improved symptoms (Jaswal et al., 2003). Similarly antioxidants and few fatty acids have been suggested to ameliorate RA and related disorders (Darlington and Stone, 2001). It was reported that intake of certain antioxidant micronutrients like β-cryptoxanthine, supplemental zinc and a diet rich in fruits and cruciferous vegetables protected against the development of RA (Cerhan et al., 2003; Pattison et al., 2005).

1.12.6 Lysosomal instability and rheumatoid arthritis

Lysosomes are small intracellular organelles present in most animal cells of widely different evolutionary development. Lysosomes in different cell types contain different enzymes and bioactive molecules. All lysosomes contain an array of hydrolytic enzymes which hydrolyze proteins, nucleic acid, polysaccharides and phospholipids.
It was suggested by Dingle (1962) that lysosomal enzymes might be responsible for damage to cartilage matrix in RA. The altered activities of these hydrolytic enzymes occur during arthritis due to the metabolic need of the cell to degrade various constituents such as mucopolysaccharides and glycoproteins accumulated in tissues due to arthritis associated with vasculopathies (Weissmann, 1972). Studies have shown that enhanced prostaglandin, thromboxane and leukotriene production during arthritis increase the extracellular activities of lysosomal enzymes like acid phosphatases, cathepsin and other collagenolytic enzymes and cause destruction of glycosaminoglycans and glycoproteins in cartilage (Anderson 1976; Weisman 1972).

Arumugham and Bose (1980) reported increased activities of lysosomal glycohydrolases *viz.* P-glucuronidase, β-N-acetyl glucosaminidase, β-galactosidase, α-mannosidase, acid phosphatase and cathepsin D in the liver of adjuvant induced rats. These enzymes contributed to tissue damage and necrosis of hepatic tissue and also aggravated inflammation all over the body (Arumugham and Bose, 1980).

### 1.12.7 Immune response during rheumatologic conditions

Stuart and Dixon (1983) stated that pathogenesis of arthritis was antibody driven. There were reports stating that CII and CpG motifs or immune stimulatory DNA sequences (*palindromic sequences of unmethylated CpG dinucleotides that are present in bacterial DNA, but not in mammalian DNA*) (ISS) are critical factors that contribute to the severity of inflammation in arthritis (Trentham et al., 1977; Giant et al., 1987).

Previous reports have shown that RA patients possess autoantibodies to Fc fraction of Immunoglobin G (IgG) in their blood (Nordlin and Klareskeg, 1988; Krieg, 2000). Enhanced formation of IgG-collagen type II complexes in RA joints together with T cell activation constitute the basis of rheumatoid factor (RF) formation (Nordlin and Klareskeg, 1988). Such complexes stimulate the expression of co-stimulatory molecules and the production of cytokines like interleukin-12, TNF-α and interferons by macrophages, dendritic cells, B cells and natural killer cells (Krieg, 2000) leading to a skew up an immune response towards a strong and prolonged T helper 1 (Th$^1$) type of immunity (Chu et al., 1997). This Th$^1$ / Th$^2$
imbalance could be a mechanism for RA perpetuation (Davis, 2004). It was discovered that rodents with haplotypes H-2q and H-2r, when induced with collagen induced arthritis generated anti-CII antibodies (Stuart and Dixon, 1983). Interestingly, normal rats injected with sera from CIA rats also developed arthritis (Stuart and Dixon, 1983). These studies provided evidence of the role of antibodies in arthritis.

1.13 Therapies for rheumatoid arthritis

Drugs to suppress inflammatory response and ameliorate damage to articular cartilage are currently used to treat arthritis. A number of therapies have been utilized in the medical management of arthritis to achieve these goals.

1.13.1 Non-steroidal anti-inflammatory drugs

NSAIDs are the most widely used medication to effectively control inflammation and pain in arthritic disorders. In 1971, Vane demonstrated that aspirin and related NSAIDs inhibit COX. NSAIDs exerts a variety of effects like anti-inflammatory, antipyretic and analgesic actions by inhibiting the synthesis of PGs by COX (Raskin, 1999) (fig. 1.17).

![Cell membrane phospholipids](Fig. 1.17 Inhibition by different classes of anti-inflammatory drugs)
However, long term use of most NSAIDs by RA patients results in adverse effects like abdominal pain, diarrhea, nausea and the more serious side-effects like bronchospasm (Jaggi et al, 2004). Studies have showed that Naproxen, Ibuprofen, Indomethacin and Nimesulide significantly inhibited matrix synthesis and produced toxic effects on cartilage metabolism (Dingle, 1999). Other drawbacks of NSAIDs include interference with bone repair and remodeling (Jaggi et al, 2004), disturbed sleep pattern (Murphy et al., 1994), increased insulin secretion (Metz et al., 1991) as well as kidney and cardiovascular complications (Raskin, 1999; Ahmad et al., 2002).

Another critical disadvantage of NSAID usage is the preferential inhibition of COX-1 in gastric mucosa resulting in alteration of homeostatic functions of the stomach (Vane and Botting, 1998). The inhibition of COX-1 or prostaglandin synthesis by NSAIDs causes increased production of leukotrienes by 5-LOX. This is because COX isozymes and 5-LOX share the same substrate arachidonic acid and inhibition of the COX pathway leads to a shift towards the production of leukotrienes by 5-LOX due to increased substrate availability which also induce the formation of gastric ulcers (Peskar et al., 1991; Hudson et al., 1993).

1.13.2 Disease modifying anti-rheumatic drugs (DMARDs)

The traditional treatment of RA is represented by a pyramidal approach starting with non-steroidal anti-inflammatory drugs and progressing to so-called disease modifying anti-rheumatic drugs such as gold, sulphasalazine, leflunomide and methotrexate (Sharp et al., 1991). It was found that early therapy with DMARDs resulted in better long-term outcome in arthritic patients than late therapy (Lard et al., 2001). Kumar and Marwaha (2003) reported that combination treatments were also well tolerated and result in better outcome than monotherapy. Kremer et al. (2000) reported that a combination of leflunomide with methotrexate was superior in modulating arthritic progression compared to methotrexate alone (Kremer et al., 2000).
1.13.3 **COX-2 inhibitors**

COX-2 inhibitors are second generation of anti-inflammatory drugs developed to specifically target COX-2 induced prostaglandin production so that the homeostatic functions of COX-1 is not disturbed (Chan et al., 1995; Hay et al., 1997; Khanna et al., 1997; Zhang et al., 1997). However, while reducing the risk of gastropathy, research indicated that COX-2 specific inhibitors were not free of adverse side-effects. After reviewing the results of several randomized trials with COX-2 inhibitors including the VIGOR (Vioxx gastrointestinal outcomes research) (Bombardier, 2000) and CLASS (Celecoxib long term arthritis safety study) (Silverstein et al, 2000), it was concluded that a potential increase in cardiovascular event rates was associated with the continuous use of these agents (Mukherjee et al, 2001).

Furthermore, it was reported that selective COX-2 inhibitors cause adverse renal effects like sodium, potassium and water retention as well as decreased renal function, similar to those of conventional non-selective NSAIDs (Hudson et al., 1993; Ahmad et al., 2002). Interestingly, Soni et al. (2009) recently reported the hepatic safety and tolerability of a COX-2 selective NSAID celecoxib.

1.13.4 **Immunosuppressants**

The glucocorticoids possess immunosuppressive and anti-inflammatory activities. Glucocorticoids derivatives like NCX-1015 were reported to alleviate experimentally induced arthritis by elevating bone resorbing activity (Clark et al., 2002). In animal models, immunization with T cell receptor (TCR) Va, an immunosuppresant chains blocked the development of inflammation, synovial hyperplasia and erosion of bone and cartilage (Haqqi et al., 1996). An alternative therapeutic strategy was the use of lipoxin receptor agonists which negatively modulates cellular and humoral response. BML-111, a lipoxin receptor agonist modulated arthritis by its immunomodulatory effect (Zhang et al., 2008).

1.13.5 **Anti-cytokine therapy**

Anti-cytokine or biological DMARDs target downstream inflammatory mediators like TNF-α and IL-6. Examples include infliximab, etanercept and
adalimumab [IL-1 (anakinra)] (O’Dell, 2004). Kumar and Marwaha (2003) reported that Etanercept, a high affinity type II TNF-α receptor covalently linked to the Fc portion of IgG1 brought about 40-60% improvement in RA patients. Infliximab, a chimeric mouse / human anti-TNF-α monoclonal antibody (mAb) binds and inactivates soluble TNF-α. Adalimumab is a recombinant human IgG1 monoclonal antibody specific for TNF-α. Anti-IL-6 mAbs, inhibitors of IL-6 mRNA expression and IL-6 receptor were also effective in decreasing RA (Miyazawa et al., 1998; Takagi, 1998; Henningan and Kavanaugh, 2008). IL-11 also gained attention as anti-inflammatory agent due to its ability to decrease TNF-α, MMP-1 and MMP-3 activities and reduced joint destruction (Hermann et al., 1998).

1.13.6 NF-κB-directed therapy

NF-κB-directed therapy has been found effective in animal model of arthritis. NF-κB blockade with NF-κB decoy oligonucleotides through injection or viral gene transfer was found to inhibit collagen induced and streptococcal wall induced arthritis in rats (Miagkov, 1998).

1.13.7 Angiogenesis inhibitors

Intraarticular administration of rabbits with cyclic peptide antagonist of v3 induced vascular apoptosis decreased pannus formation, synovial infiltration and joint swelling (Storgard et al., 1999).

1.13.8 Gene therapy

Adenovirus mediated transfer of death factor Fas/Apo and its ligand (9Fas L) was reported to enhance Fas L expression in mice which induced apoptosis of synovial cells thereby leading to reduced progression of disease (Roessler et al., 1993).

1.13.9 Physical treatments for rheumatoid arthritis

The main goal of physical therapy is to increase the muscular strength around affected parts and maintain joint range of motion. Heat is normally employed. Cryotherapy is also employed to mitigate acute pain by reducing the temperature of neural receptors. Physical therapy is also reported to play recovery
role in patients who underwent total hip arthroplasty and total knee arthroplasty (Ganz and Harris, 1998).

1.13.10 Natural products in treatment of rheumatoid arthritis

Natural products act through diverse mechanisms to alleviate arthritis (Taibi and Bourguignon, 2003). In patients with autoimmune diseases, the use of dietary supplements was on the rise mainly because they are effective, inexpensive and readily safe (Kumazawa, 2006). A number of vegetable oils have been claimed to provide benefit in rheumatoid arthritis. Cleland et al. (1988) found improvement in RA patients taking olive oil for 14 weeks. Improvement was also seen in RA patients consuming evening primrose oil rich in γ-linoleic acid (GLA) (Brzeski et al., 1991). Watson et al., 1993 reported that morning stiffness in RA patients was significantly ameliorated after taking black currant seed oil (rich in GLA). The monocytes cultured from these patients exhibited a lower secretion of inflammatory cytokines like IL-1, IL-6 and TNF-α when compared with control subjects (Watson et al., 1993).

Mulberry extracts rich in anthocyanins, glucosides from Chaenomeles speciosa and Paeony remitted adjuvant induced arthritis by ameliorating inflammation and inhibiting synoviocyte activities (Dai et al., 2003; Kim and Park, 2006; Xu et al., 2007). Betula platyphylla extract inhibited degradation of prostaglandin and collagen in cartilage explants (Cho et al., 2006). Green tea polyphenols inhibited COX-2, NOS and MMP activities in human chondrocytes in vitro (Ahmed et al., 2002; Ahmed et al., 2004). Cyclosporin, a fungal metabolite was reported to inhibit arthritis by its immunosuppressive effects (Takagishi et al., 1986). Joe and Lokesh (1997) demonstrated that curcumin and capsaicin lowered release of lysosomal membranes and eicosanoid production in rat peritoneal macrophages. Recently it was reported that stabilized rice bran extracts modulated COX-1, COX-2 and 5-LOX activities and has application as a functional food (Rosche et al., 2009). Studies have shown that rats supplemented with Kalpaamrutha, a herbal preparation and leaf extracts from Cleome gyandra L. showed improvement in arthritis due to its lysosome stabilization effects (Narendhirakannan et al., 2007; Mythilypriya et al., 2008 a, b). Thus natural
products especially the Ayurvedic drugs alleviate inflammatory diseases through multiple mechanisms (fig. 1.18).

**Figure 1.18 Molecular targets of Ayurvedic drugs.** AP: Activated protein; COX: Cyclooxygenase; IAP: Inhibitor of apoptosis protein; ICAM: Intercellular cell adhesion molecule; JAK: Janus kinase; MDR: Multi-drug resistance; MMP: Matrix metalloproteinase; NF-κB: Nuclear factor kappa B; STAT: Signal transducer and activator of transcription; VEGF: Vascular endothelial growth factor; XIAP: X-linked inhibitor of apoptosis (Aggarwal et al., 2006).

1.14 *Bacopa monniera* (L.) Wettst

*Bacopa monniera* also referred to as Bacopa monnieri, *Herpestis monniera*, water hyssop, thyme leafed gratiola and Brahmi is a member of the Scrophulariaceae family. It is a small, creeping herb with numerous branches, small oblong leaves, and light purple flowers (fig 1.19). It commonly grows in marshy areas throughout India, Nepal, Sri Lanka, China, Taiwan and Vietnam. It is also found in Florida and other Southern states of the USA where it is grown in damp conditions like pond or bog garden. The herb can be found at elevations from sea level to altitudes of 4,400 feet, and is easily cultivated if adequate water is available. Flowers and fruit appear in summer and the entire plant is used medicinally (Chopra, 1958; Bone, 1996).
Bacopa monniera has been used by Indian Ayurvedic medical practitioners for almost 3000 years and is classified as a Medhya rasayana, a drug used to improve memory and intellect (Mukherjee and Dey, 1996; Husain et al., 2007). It is also used to provide relief to patients with anxiety or epileptic disorders (Chopra, 1958). In Pakistan, Bacopa is used as a cardiac tonic. It is also used to treat digestion problems and to improve respiratory function in cases of bronchoconstriction (Nadkarni, 1988).

Bacopa monniera has been introduced in the Indian market and in other countries, alone or in combination with other phytocomplexes and utilized in the treatment of memory and inflammatory disorders. Brahmi Ghrita, a polyherbal formulation containing Bacopa monniera, Evolvulus alsinoids, Saussurea lappa and cow’s ghee is used in Ayurveda as memory enhancer and anti-convulsant (Achliya et al., 2004). Brahmi Rasayan with Bacopa monniera as an active constituent is used to treat inflammatory disorders (Jain et al., 1994).

Most research on Bacopa monniera has focused on its cognitive modulating abilities. The triterpenoid saponins in this herb - the bacosides are responsible for the ability of Bacopa monniera to enhance nerve impulse transmission. The constituents found to be responsible for Bacopa monniera’s cognitive effects are bacosides A and B (Mahato et al., 2000; Chakravarty et al., 2001).
The traditional use of *Bacopa monniera* as an anti-anxiety remedy in Ayurvedic medicine was supported by both animal and clinical research. Studies have shown that *Bacopa monniera* extract (standardized to 25% bacoside A) exerted anxiolytic activity comparable to Lorazepam, a common benzodiazepine anxiolytic drug. The extract did not induce amnesia or side effects associated with Lorazepam, but instead had a memory-enhancing effect (Bhattacharya and Ghosal, 1998).

Studies have demonstrated the effects of *Bacopa monniera* extracts on the gastrointestinal tract (Dar and Channa, 1999). *Bacopa monniera* healed acetic acid induced ulcers and strengthened the mucosal barrier and decreased mucosal exfoliation (Sairam et al., 2001).

Previous studies have demonstrated that bacosides exhibited antioxidant properties. Bacosides improved antioxidant activity in the hippocampus, frontal cortex and striatum in brain by modulating the expression of certain enzymes involved in generation and scavenging of reactive oxygen species in this organ (Bhattacharya et al., 2000; Chowdhuri et al., 2002). The extract also alleviated stress-induced ulcers by significantly reducing lipid peroxidation and improving antioxidant status in rat gastric mucosa (Sairam et al., 2001). *Bacopa monniera* also decreased the levels of malondialdehyde in prostrate gland of aging mice (Kalamade et al., 2008). An important property of *Bacopa monniera* recently
published was the reversible suppression of spermatogenesis and fertility in rodents (Sing and Singh, 2009).

In vivo studies have demonstrated that *Bacopa monniera* extract exert protective effects against certain drugs and their negative side effects. *Bacopa monniera* reduced morphine induced withdrawal effects in rats (Sumathi et al., 2002). Administration of *Bacopa monniera* extract with morphine significantly decreased lipid peroxidation and increased levels of antioxidant enzymes and glutathione in rat hepatic tissue, when compared to morphine alone treated rats. Previous works from our laboratory showed that *Bacopa monniera* exerted hepatoprotective and genoprotective effects against nicotine, a content of cigarette smoke (Vijayan and Helen, 2007). These results suggest a protective effect of *Bacopa monniera* on the antioxidant status in drug-treated rats.

In mice, administration of *Bacopa monniera* with phenytoin, an anti-epileptic drug significantly reversed phenytoin-induced cognitive impairment, as noted by improved acquisition and retention of memory. These results suggest a potential corrective effect of *Bacopa monniera* extracts in phenytoin-induced cognitive deficit (Vohora et al., 2000).

In vitro and in vivo studies by Dar and Channa (1997 and 1999) demonstrated the broncho vasodilatory activity of *Bacopa monniera* extract on rabbit and guinea pig trachea, pulmonary artery and aorta. Another study demonstrated that *Bacopa monniera* possessed potent mast cell stabilizing activity comparable to disodium cromoglycate, a commonly used allergy medication (Samiulla et al., 2001). These studies indicate the potential usefulness of Bacopa extracts in bronchoconstrictive and allergic conditions.

In vitro research demonstrated that saponins from *Bacopa monniera* inhibited DNA replication and thus has cytotoxic activity on sarcoma-180 cells (Elangovan et al., 1995). At the same time *Bacopa monniera* exerted a protective effect against DNA damage in astrocytes (Russo et al., 2003b) and human fibroblasts (Russo et al., 2003a). A recent study showed that *Bacopa monniera* extract induced apoptosis in murine sarcoma cells (Rohini and Devi, 2008).
Bacopa monniera has been used safely in Ayurvedic medicine for several hundred years. Reports have shown that therapeutic doses of Bacopa monniera extract do not cause any known side-effects. Concentrated bacosides given in single (20-30 mg) and multiple (100-200 mg) daily doses to healthy volunteers were well tolerated without adverse effects (Singh and Dhawan, 1997). The LD$_{50}$ of Bacopa extracts administered orally to rats was 5 g / kg for aqueous extract and 17 g / kg for alcohol extract. Neither extract resulted in gross behavioral changes at these concentrations (Martis and Rao, 1992).

Compounds responsible for the pharmacological effects of Bacopa monniera include alkaloids, saponins, and sterols. Many active constituents, the alkaloids Brahmine and herpestine, saponins d-mannitol and hersaponin, acid A and monnierin were isolated in India over 40 years ago. Other active constituents have since been identified are betulinic acid, stigmasterol, $\beta$-sitosterol, as well as numerous bacosides and bacopasaponins (Jain et al., 1994).

### 1.15 Objectives of the present study

A brief review of literature given above indicates that inflammation plays a crucial role in the pathogenesis of inflammatory diseases like arthritis. Improper activation or upregulation of pro-inflammatory mediators during arthritis paves way for inflammation in the synovium and destruction of cartilage and underlying bone. Despite the extensive amount of evidence in support of inflammation and the preventive potential of several anti-inflammatory drugs currently available in the market, more research is needed to screen and evaluate the mode of action of natural drugs with minimal or no side effects. This is essential because each individual is unique and respond differently to different drugs. Therefore, the identification of naturally occurring phytocompounds that suppress or downregulate the function of pro-inflammatory mediators can lead to the discovery of important anti-inflammatory therapeutics.

It has been proposed that edible and medicinal plants possess substantial anti-inflammatory properties. In light of this, we selected Bacopa monniera (L.) Wettst for this study. This plant is described in the Ayurvedic texts as a wonder drug used to cure many ailments. In the midst of the many pharmacological
properties exhibited by this plant, it was of interest to us to study the anti-inflammatory effects of this herb as this is anecdotally used in the treatment of rheumatism. The anti-inflammatory property of this plant was recently demonstrated (Channa et al., 2006) but the cellular and molecular mechanisms underlying the anti-inflammatory property of *Bacopa monniera extract in vivo* is currently not well defined. Another criterion for selecting this plant is that it is easily available; it can be easily propagated and is also edible. The broad objectives of the present study were to:

- Screen the different extracts of *Bacopa monniera* for anti-inflammatory property.
- Study the effect of the most potent extract in acute and chronic inflammation models.
- Examine the anti-arthritic effects of *Bacopa monniera* extract.
- Isolate the active compound by bioactive guided fractionation and study its anti-arthritic potential.
- Identify the active component responsible for anti-inflammatory activity.
- Study the anti-inflammatory activity of the isolated component and elucidate the mode of action of this compound.

The results of these investigations have been discussed in the following chapters.