Introduction & Review of Literature
I.1 Rheumatoid Arthritis as a Disease

Rheumatoid arthritis (RA) is an autoimmune disease, characterized by chronic inflammation in the joints and subsequent destruction of cartilage and bone. Rheumatoid arthritis affects 0.5 to 1% of the population worldwide.\textsuperscript{1,2} Geographic and ethnic variations exist in terms of propensity and manifest symptoms of the disease. A high prevalence has been found among North American Indians (3.5-5.3%) and a low prevalence has been reported in rural South African blacks and in Japanese (0.1%).\textsuperscript{3} It is a disease with clear gender bias: women are affected three times as often as men. It is associated with reduced life expectancy and is a major cause of chronic disability and handicap. The etiology of RA is still unknown but it has been recognized that many cytokines are up-regulated in RA synovium\textsuperscript{4,5} which are correlated with the joint lesions.\textsuperscript{6,7} This disease can occur at any age, but it is most common among 40-70 years age group and the incidence increases with age. Diagnosis is heavily reliant on the presence of particular clinical features rather than laboratory investigation (Table 1). Yet diagnostic criteria have been developed by the American College of Rheumatology.\textsuperscript{8}

I.2. History of Rheumatoid Arthritis

The history of rheumatoid arthritis is quite old. The earliest known appearance of RA was noted in the skeletal remains of Indians from 4500 BC found in what is now known Tennessee and the first written reference to arthritis description was found in the Charaka Samahita, an Indian text from 123 AD.

Evidences of RA in Europe first appeared in early 17\textsuperscript{th} century. In contrast, skeletal remains suggest that the disease has existed in North America for at lest 3000 years. Sydenham published the first case report in 1676 although intermittent case series were subsequently reported. The disease was not fully defined until 1859. It was Sir Alfred Garrod, a London based physician who coined the clinical term 'rheumatoid arthritis' and the first reference in modern medical literature.

Until 1957 RA was viewed as separate from osteoarthritis. Later Chares Short described RA definitively and clearly set it apart as a defined clinical entity distinct from the
Table 1. American College of Rheumatology (ACR) criteria for rheumatoid arthritis

1. Morning stiffness lasting for more than one hour for more than six weeks
2. Arthritis in three areas for more than six weeks
3. Arthritis of the hands or wrists for more than six weeks
4. Symmetrical arthritis for more than six weeks
5. Rheumatoid nodules
6. Positive test result for rheumatoid factor
7. Radiographical changes in the wrists/hands

Four criteria are required for the diagnosis of rheumatoid arthritis
seronegative spondyloarthropathies, crystal-induced disease, osteoarthritis, systemic lupus erythematosus and many other conditions. 

1.3. Pathogenesis

Pathogenesis of RA is quite complicated. RA primarily affects the small diarthroidal joints of the hand and feet. Chronic inflammation of the joints result in redness, swelling, pronounce pain in the joints and finally causes disability and handicap. In RA, the major site of tissue destruction originates at the junction of the synovium lining the joint capsule with cartilage and bone (Fig 1.1). Normally in the knee joint, the synovium consists of a synovial membrane (usually one or two cells thick) and underlying loose connective tissue. Synovial-lining cells are designated type-A (macrophage-like synoviocytes) or type-B (fibroblast-like synoviocytes). In early rheumatoid arthritis, the synovial membrane becomes thickened because of hyperplasia and hypertrophy of the synovial lining cells. An extensive network of new blood vessels is formed in the synovium. T cells (predominantly CD4+) and B cells (some of which become plasma cells) infiltrate the synovial membrane. These cells are also found in the synovial fluid, along with large numbers of neutrophils. In the early stages of rheumatoid arthritis, the synovial membrane begins to invade the cartilage. In established rheumatoid arthritis, the synovial membrane becomes transformed into inflammatory tissue, the pannus. This tissue invades and destroys adjacent cartilage and bone. The pannus consists of both type A and type B synoviocytes and plasma cells. The cells of the pannus migrate over the underlying cartilage and into the subchondrial bone, causing the subsequent erosion of these tissues.

Early theories on the pathogenesis of rheumatoid arthritis focused on autoantibodies and immune complexes. The first clue that self-reactivity plays a key role in RA was the identification of ‘rheumatoid factor’ in the blood of affected patients. Rheumatoid factor was observed originally by Waaler in 1939 and later rediscovered by Rose by virtue of its ability to agglutinate sheep red cells that had been coated with rabbit serum. The seminal studies of Kunkel ultimately characterized the unknown factor as an antibody that binds to Fc portion of immunoglobulins. This observation led to the logical view that RA might be an autoimmune disease caused by self-reactive antibodies. The primary pathogenic potential of rheumatoid factor in RA as an initiator of immune
Fig.1.1. Pathogenesis of Rheumatoid Arthritis.
complex-mediated disease was formulated during the 1960s. In this model, immune complexes formed by rheumatoid factors and perhaps other autoantibodies fix complement and release chemotactic factor such as C5a. Inflammatory cells are subsequently recruited to the rheumatoid joint along a chemotactic gradient where they are activated and contribute to local destruction. Neutrophils, in particular, accumulate in synovial fluid where they engulf immune complexes and release proteolytic enzyme.

Though the etiology of RA is still unknown but most investigators agree that the chronic joint inflammation in RA (Fig1.2) is induced by antigen-stimulated T cells infiltrating the synovial membrane. A few well documented clues suggest arthritis to be associated with certain MHC (HLA) class II genes (Table1.2) those encoding the β chain of the DR1 and DR4 molecules which confer inherited susceptibility to RA and the tissue specificity of inflammatory attack. On one side of peptide binding groove of HLA-DR there is a shared epitope, comprising amino acids 70-74 of the β chain, which is conserved in the DR1 and DR2 disease susceptible haplotypes. This is the most important evidence to support the concept that T-lymphocyte recognition is important at some stage in pathogenesis of rheumatoid arthritis, either in shaping the T-cell receptor (TCR) repertoire or in presentation of an inducing microbial or auto antigenic peptide. These observations strongly suggest that autoimmune T-cell dependent mechanisms are important in the pathogenesis of rheumatoid arthritis. One possibility is that the recognition structures are autoantigens, presumably located in the peripheral joints, which are main targets of inflammation in rheumatoid arthritis. Support of this assumption is the frequent occurrence of antibody to cartilage component in rheumatoid arthritis

The inflammatory cells, including the T cells, enter the synovial membrane via high endothelial venules and the expression of adhesion molecules leukocyte function-associated antigen-1 (LFA-1), intercellular adhesion molecule-1 (ICAM-1), and chemokines and their receptors facilitates this cell traffic.

Nitric oxide (NO) is synthesized by a family of nitric oxide synthases and is produced by all kinds of cells. IL-1, TNF-α, and interferon-gamma (IFN-γ) up-regulate the activity of certain nitric oxide synthases and cyclooxygenase-2 (COX-2), leading to augmented production of prostaglandins (PG) and NO. Nitric oxide has many catabolic
Fig. 1.2. Cells and molecules involved in the initiating immune response and the subsequent inflammation. TCR: T-cell receptor for antigen; APC: Antigen presenting cell; HLA: Human leukocyte antigen. PMN’s: Polymorphonuclear cells. NO: Nitric oxide; PG: Prostaglandins; CD: Cluster of differentiation. (Ref. Førre, Ø., et al., Scandi. J. Rheumatol. 2000; 29; 73-84.)
Table 1.2. Potential role of HLA-DR in RA

- Binds to arthritogenic peptides that can be presented to T cells
- Shapes the T-cell repertoire and permits escape from tolerance or survival of autoreactive clones.
- Leads to enhanced T-cell reactivity owing to unique contacts between T-cell receptors and MHC molecules.
- Serves as a target for autoreactive T cells owing to molecular mimicry with a pathogen (for example, Escherichia coli DnaJ or Epstein–Barr virus peptides)
- Closely linked to other genes in the MHC that are associated with RA
- Fails to bind peptides of an arthritogenic pathogen, leading to an inadequate immune response

(Ref. Firestein, G.S. Nature 2003, 423,356-361)
effects on chondrocyte functions in RA. It activates metalloproteases, down-regulates synthesis of proteoglycans and collagen II, inhibits PGE2- production, increases apoptosis, and down-regulates IL-1 receptor antagonist.

The inflammatory process leads to:

1. Production of immunoglobulins such as rheumatoid factors with formation of immune complexes resulting in complement activation.
2. Synovial cell proliferation with production of various matrix-metalloproteinases (MMPs), which degrade extracellular matrix component and type II collagen.21
3. Formation of new blood vessels (neovascularization) induced by macrophage and fibroblast derived growth factors, cytokine, and chemokines.22,23
4. Formation of pannus, a vascular granulation tissue, which attaches to the surface of articular cartilage and bone via adhesion molecules.

The analysis of the mRNA for cytokine of rheumatoid joint revealed the abundant expression of proinflammatory cytokines and chemokines e.g., TNF-α, IL-1, IL-6, GM-CSF and IL-8. The analysis of cytokines expression and regulation yield effective therapeutic targets in inflammatory diseases. TNF-α is one of the initiator and regulator of inflammatory response. It has been well documented that there is ectopic expression of this cytokine in rheumatoid joint. Its expression at synovial joint is correlated with the progression of rheumatoid arthritis. TNF-α is identified as the major regulator in rheumatoid arthritis, which acts at the apex of the cascade of reactions resulting in destruction of cartilage and bone. This suggests TNF-α as the prime target for therapeutic trials.5

I.4. Experimental Models of Rheumatoid Arthritis

Animal models of autoimmune diseases have been developed for a number of human disorders. The rationale for developing animal models is to provide tools to identify the inciting antigen, define the molecular and cellular events that lead to clinical disease and test the efficacy of therapeutic strategies. While none of the current models reflects the full complexity of human disease, many models have been useful in providing
insights into the biological mechanisms underlying rheumatoid arthritis. There are several mouse models reviewed in table 1.3 to study rheumatoid arthritis.

1.4.1. Induced Arthritis Models

Multiple triggering agents can induce arthritis in genetically susceptible strains of inbred mice. Such agents can be live bacteria or bacterial components\textsuperscript{24-26}, oils\textsuperscript{27}, ubiquitous antigens\textsuperscript{28} or cartilage specific proteins\textsuperscript{29-31}.

Although the nature of the autoantigens in RA is not fully understood, joint specific antigens such as collagen type II and proteoglycan are possible candidates. Immunization with collagen type II or the major cartilage proteoglycan, aggrecan, induce arthritis in some inbred strains of mice. Proteoglycan-induced arthritis (PGIA) is inducible in mice using heterologous antigen, such as human cartilage proteoglycan\textsuperscript{32}. PGIA has been reported to be a T-cell-mediated disease and polygenically controlled\textsuperscript{32}.

The most commonly used model for RA in mice, however, is CIA\textsuperscript{33}, which has been extensively studied for more than two decades, CIA is induced by intradermal injections of collagen type II together with adjuvant. This will be discussed in details in the next section. Other mouse models for arthritis are pristane induced arthritis, adjuvant-induced arthritis and proteoglycan induced arthritis.

1.4.2. Spontaneous Models of Arthritis

There are several examples of genetically manipulated mouse strains that develop arthritis (Table 1.3.). One recently described model is the spontaneously triggered arthritis in K/B×N mice\textsuperscript{34}. This model is generated by crossing an autoimmune prone non obese diabetic (NOD) mouse and a C57BL/6 mouse transgenic for a TCR recognizing bovine ribonuclease peptide. The offspring develop spontaneous arthritis at 3 to 4 weeks age. The diseases can passively be transferred to a healthy mouse with serum of arthritic mice\textsuperscript{35}, which indicates that Abs or some serum factor is important for the development of arthritis.
Table 1.3. Models for rheumatoid arthritis.

<table>
<thead>
<tr>
<th>Model type</th>
<th>Disease name</th>
<th>Induction agent/target for genetic manipulation</th>
<th>Disease characteristics</th>
<th>MHC dependence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Induced Arthritis Models</td>
<td><em>Staphylococcus aureus</em> induced arthritis</td>
<td>Toxic shock syndrome toxin-1 producing S. aureus LS-1 strain</td>
<td>Acute</td>
<td>H2^b&gt;H2^a,H1^a</td>
</tr>
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<td></td>
<td><em>Borrelia burgdorferi</em>-associated arthritis</td>
<td>Borrelia burgdorferi</td>
<td>Acute</td>
<td>Nd</td>
</tr>
<tr>
<td></td>
<td>Collagen- induced arthritis</td>
<td>Heterologous collagen type II</td>
<td>Acute/chronic</td>
<td>H2^a = H2^b&gt;H2^k</td>
</tr>
<tr>
<td></td>
<td>Collagen- induced arthritis</td>
<td>Homologous collagen type II</td>
<td>Chronic</td>
<td>H2^a</td>
</tr>
<tr>
<td></td>
<td>Proteoglycan-induced arthritis</td>
<td>Human cartilage proteoglycan</td>
<td>Acute to chronic</td>
<td>H2^a, H2^k</td>
</tr>
<tr>
<td></td>
<td>Pristane-induced arthritis</td>
<td>Pristane</td>
<td>Chronic</td>
<td>Nd</td>
</tr>
<tr>
<td></td>
<td>Anti-CII antibody-induced arthritis</td>
<td>Transfer of collagen type II specific antibodies</td>
<td>Acute</td>
<td>MHC independent</td>
</tr>
<tr>
<td></td>
<td>Anti-GPI antibody transfer-induced arthritis</td>
<td>Transfer of GPI- specific antibodies</td>
<td>Acute</td>
<td>MHC independent</td>
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<thead>
<tr>
<th>Genetically Manipulated Models</th>
<th>TNF- alpha transgenic</th>
<th>Human tumor necrosis factor</th>
<th>Acute</th>
<th>Nd</th>
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<tr>
<td>HTLV-1 env^* pX transgenic</td>
<td>IL-1Ra^*</td>
<td>IL-1 receptor antagonist</td>
<td>Chronic</td>
<td>Nd</td>
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<tr>
<td>K/B=N TCR transgenic</td>
<td></td>
<td>Human T-cell Leukemia Virus Type 1 env^* pX</td>
<td>Chronic</td>
<td>MHC independent</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H2k restricted bovine ribonuclease (KRN) peptide specific TCR</td>
<td>Chronic</td>
<td>H2^b</td>
</tr>
</tbody>
</table>

| Environment Induced Models    | Stress-induced arthritis |                                          | Chronic                 | Nd                   |

*Ref. Holmdahl R. et al. Trends in Genetics 2002*
**I.4.3. Collagen Induced Arthritis**

Collagen induced arthritis (CIA) was first reported by Trentham DE and colleagues in the year 1977. They showed that intradermal injection of native type II collagen induces an inflammatory arthritis in 40% of several strains of rats later similar pathology was reported in primates and genetically susceptible strains of mice.

CIA is induced by interadermal injection of homologous or heterologous (bovine, chick or rat origin) type II collagen in complete Freund’s adjuvant (CFA) and involves the activation of both T and B cells. In rats, both CFA or incomplete Freund’s adjuvant and CII can trigger arthritis while in mice CFA, which contains attenuated *Mycobacterium tuberculosis*, is needed. Mice immunized with CII/CFA result in polyarthritis with visible clinical symptoms, redness and swelling of the paws, after 3-4 weeks.

The histopathology of mouse arthritis joints resemble the abnormalities observed in RA patients, such as synovial hyperplasia, mononuclear cells infiltration, pannus formation and cartilage damage and bone erosion. CIA is MHC dependent and is characterized by erosive joint inflammation mediated by both T and B cells. Studies in this model demonstrated that collagen-specific T cells capable of autoaggression are present in the periphery of healthy individuals.

The susceptibility to CIA is genetically linked with MHC locus and may be linked to T-cell receptor (TCR) haplotypes. CIA susceptibility is specifically associated with the I-A region of the H2q and H2r haplotypes in mice. A bottleneck in the development of disease is the T cell recognition of an immunodominant CII-derived peptide bound to MHC class II with a certain affinity. The critical importance of a single MHC class II gene was shown in a transgenic mouse model for arthritis. The ‘q’ haplotype of a mouse MHC (H2q) but not the ‘p’ haplotype (H2p) is associated with susceptibility to CIA in mice. However, by introducing a change in the H2p resulting in four amino acids changes of the beta chain of MHC class II A molecule in transgenic mice, it mimics exactly the beta chain of corresponding molecules in the H2q haplotype. The mice carrying this transgene were now susceptible to CIA.
I.5. Role of Cytokines in RA

Cytokines are a group of low molecular weight regulatory proteins secreted by white blood cells and a variety of other cells in the body in response to a number of inducing stimuli. Cytokines bind to specific receptors on the membrane of target cells, triggering signal transduction pathways that ultimately alter gene expression in the target cells. In general, the cytokines and their receptors exhibit very high affinity for each other with dissociation constant ranging from $10^{-10}$ to $10^{-12}$ M. Because of this high affinity, picomolar concentrations of cytokines can mediate a biological effect.

Cytokines play important role in development of an effective immune response but under certain pathological condition their up-regulation results in progression of disease. In RA there is up-regulation of the cytokines that leads to progression of pathogenesis (Fig. 1.3). Analysis of the mRNA for cytokines of rheumatoid joint revealed abundant expression of proinflammatory cytokines e.g., TNF-α, IL-1, IL-6, GM-CSF and IL-8. T cell products as IL-3, IL-4, IFN-γ, TNF-β, and IL-2 are present in low concentrations. Both TNF-α and IL-1 are likely to have primary role in the pathogenesis of rheumatoid arthritis. The serum and synovial concentration of both cytokines are high in patients with active rheumatoid arthritis. Furthermore, TNF-α and IL-1 are potent stimulators of mesenchymal cells, such as synovial fibroblast, osteoclasts, and chondrocytes that release tissue-destroying matrix metalloproteinase. These dual actions are thought to lead to joint damage.

IL-1 is mostly produced by monocytes and macrophages but also produced by endothelial cells, B cells, and activated T cells. The interleukin-1 signalling is more complex than TNF-α system. Interleukin-1 binds to two types of cell surface receptors. Only type I receptors have a cytoplasmic tail and are capable of intracellular signalling. Type II receptors are decoy receptors: they bind circulating interleukin-1 but do not deliver any intracellular signals. The type I receptor is found in low numbers on many cells, where as the type II receptor is expressed primarily on neutrophils, monocytes, and B cells.

Studies of arthritis in animals have strongly implicated interleukine-1 in joint damage. Injection of interleukin-1 into the knee joints of rabbits results in the degradation of
Fig.1.3. Cytokine Signalling Pathways Involved in Inflammatory Arthritis. The major cell types and cytokine pathways believed to be involved in joint destruction mediated by TNF-α and interleukin-1 are shown. Th2 denotes type 2 helper T cell, Th0 precursor of type 1 and type 2 helper T cells, and OPGL osteoprotegerin ligand.

whereas the injection of antibodies against interleukin-1 ameliorates collagen-induced arthritis in mice and decrease the damage to cartilage. Like TNF-α, interleukin-1 may cause damage by stimulating the release of matrix metalloprotinases from fibroblast and chondrocytes.

IL-6 is a pleiotropic inflammatory cytokine produced by T cells, monocytes, macrophages, and synovial fibroblast. Originally identified as a factor that induces the final maturation of B cells into plasma cells, interleukin-6 is involved in diverse biological processes, such as the activation of T cells, the induction of the acute-phase response, the stimulation of the growth and differentiation of hematopoietic precursor cells, and the proliferation of synovial fibroblast. IL-6 has been reported to contribute to the development of arthritis. IL-6 is present at very high levels in serum and synovial fluids of RA and juvenile RA patients.

I.6. Tumor Necrosis Factor Alpha

Tumor necrosis factor alpha (TNF-α), named for its ability to cause rapid necrotic tumor regression, is the founding member of the TNF ligand superfamily, which is known to have pleiotropic functions including cell proliferation, differentiation, activation, and apoptosis. TNF-α is primarily secreted by the activated macrophage and lymphocytes, neutrophils, endothelial cells, keratinocytes, and fibroblast during acute inflammatory reactions in response to bacterial and viral infections. TNF-α was isolated more than 20 years ago, on the basis of its ability to kill tumor cells in vitro and to cause hemorrhagic necrosis of transplantable tumor in mice. Concurrently, a catabolic hormone that acts as a mediator of cachexia induced by chronic infection or tumors, known as cachectin was isolated from mouse macrophages, sequenced, and shown to be identical to TNF.

In the year 1984, Aggrawal’s group purified TNF-α to homogeneity, determined the amino-acid sequence and cloned the complementary DNA, only then the true chemical identities of TNF and LT, and their relationship was revealed. The determination of amino-acids sequence of TNF indicated that the two proteins were homologous. The sequence homology between two proteins (30% amino-acids
Fig. 1.4. Some of the biological activities of TNF on different cells and tissues. (Ref. Grell, M. & Scheurich, P., 2001 Encyclopaedia of life sciences)
identity) and the existence of common cell-surface receptors led to the renaming of TNF and LT to TNF-α and TNF-β, respectively.

1.6.1 TNF-α Gene and Regulation of its Expression

The TNF-α gene is a single copy gene, closely linked within the cluster of major histocompatibility complex (MHC) genes, located on the short arm of human chromosome-6 and murine chromosome -17 at boundary of the class III and class I MHC regions. In the species examined (human, murine, and rabbit), the TNF-β is always 5' to the TNF-α gene. The TNF-α and TNF-β genes are each approximately 3 Kb long, and each gene consists of four exons and three introns. These similarities suggest that these genes were derived from a common ancestral gene by gene duplication. There is 80-90% homology between the coding regions of TNF-α and TNF-β. TNF-α can be induced by LPS-stimulation. Translational activation by LPS in a murine macrophage cell line was shown to be regulated by the UA-rich sequence in the 3' UTR of human TNF-α mRNA. The region in the TNF-α mRNA strongly suppresses the translation of a reporter coding sequence to which it was attached. The suppressive effect of UA-rich sequence on translation was also demonstrated with IFN-γ mRNA.

1.6.2 Cleavage and Release of Membrane Bound TNF-α

The precursor of the mature TNF-α is a membrane bound 26 kD protein. The cleavage at the site 76-77 amino-acid residue viz. Ala-Val results in release of 17 kD soluble, mature TNF-α which is biologically active. Most of the biological functions of TNF-α are mediated by the soluble form of this cytokine, although the membrane bound form have also been implicated in cytotoxicity. The soluble form of TNF-α binds to its cognate receptor, p55, and initiates a variety of cellular function in a wide range of cells. Metalloprotease disintegrins are known to be involved in the cleavage of TNF-α. Metalloprotease disintegrins are a family of membrane anchored glycoproteins that are comprised of several cystein protein molecules, including a pro and metalloprotease domain, disintegrin domain, cysteine domain and EFG repeat. A member of this family, TRACE (TNF-α convertase) has been identified to directly cleave TNF-α from the plasma membrane.
I.7. TNF-α as Prime Target in RA

TNF-α is one of the initiator and regulator of inflammatory response in rheumatoid arthritis and its systemic and local expression is correlated with disease progression.\(^7\)

The primary investigation involving *in-vitro* blockage of TNF-α in the synovial cultures from the rheumatoid arthritic patient’s joint tissue resulted in suppression of other cytokines specially IL-6.\(^8^2\) This was supported by concurrent work by Tony Cerami’s group, which showed that antibodies to TNF administered during infection *in vivo* reduced production of IL-1 and IL-6.\(^8^3\) These results indicated that TNF-α has a special role as a proinflammatory cytokine, as it was important in the regulation of another equally strong proinflammatory cytokine, and led to the concept of TNF-α as ‘master regulator’. Many lines of evidence support this theory including:

I. TNF-α is expressed at high levels in inflamed synovium, particularly at the cartilage-pannus junction, from RA patients.\(^4^1,4^9,8^4\)

II. Anti-TNF-α inhibits the production of other pro-inflammatory cytokines including IL-1, IL-6, IL-8, and GM-CSF.\(^4^9\)

III. TNF-α by itself, can induce joint inflammation and proliferation of fibroblast-like synoviocytes\(^8^5\), trigger cartilage destruction by inducing collagenase\(^8^6\), inhibit proteoglycan synthesis by articular chondrocytes\(^8^7\), and stimulate osteoclastogenesis and bone resorption\(^8^8\)

Pre-clinical studies have also demonstrated the importance of TNF-α in RA. In addition to the TNF-Tg mice, virtually all animal models of arthritis (i.e. adjuvant-induced, collagen-induced, and serum-induced arthritis) are ameliorated by anti-TNF-α.\(^8^2,8^9,9^0\)

Most importantly, several large multicenter, placebo-controlled, clinical trials of anti-TNF therapy for RA have shown remarkable results by decreasing inflammation, improving patient function and vitality, and attenuating cartilage and bone erosions.\(^9^1-9^3\)

Based on these studies there are now two anti-TNF treatments, etanercept (a soluble TNFR2-IgG1 fusion protein) and infliximab (a chimeric monoclonal antibody against TNF-α), that have been approved by the Food and Drug Administration and the European Medicine Evaluation Agency for RA.\(^9^4\)

Patients treated with anti TNF-α show clinical benefits (Table.1.4.)
**Table 1.4.** Clinical effects of anti-TNF therapy on biological process in RA

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**Immunology**

- Depressed immune (T-Cell) response restored.
- Reduction in rheumatoid-factor levels.

**Inflammation**

- Diminished cytokine production in joint—for example, IL-1, IL-6, TNF-α, and chemokines.
- Serum levels of vascular endothelial growth factor (VEGF), IL-1, IL-6, chemokines and acute phase proteins reduced.

**Tissue destruction**

- Diminished damage to both cartilage and bone.

**Angiogenesis**

- Reduced levels of VEGF and angiogenesis.

**Haematology**

- Reduction in elevated platelets.
- Reduction in elevated fibrinogen.
- Restoration of reduced haemoglobin.

1.8. TNF-α as a Target for Autoimmune Diseases

TNF-α and its family members present a double-edge sword. Whereas they are physiologically important cytokines required for normal responses, their inappropriate expression is harmful. TNF-α has been implicated in development of autoimmunity. TNF-α has been implicated in the pathophysiological process of both systemic lupus erythematosus (SLE) and cardiovascular disease (CVD).95

It is now becoming apparent that TNF has important role in pathogenesis of type II diabetes mellitus. TNF has been shown to interfere with an insulin-signalling mechanism by inhibiting the tyrosine kinase activities of the insulin receptor and serine phosphorylation of the insulin receptor substrate-1.96 These inhibitory effects of TNF on insulin signalling seem to be mediated through the upregulation of expression of suppressor of cytokine signalling-3 (SOCS3).97 The role of TNF has also been explored using mice in which the TNF or TNFR gene has been deleted.98 The absence of TNF resulted in markedly improved insulin sensitivity in diet-induced obesity and in the ob/ob model of obesity. The TNF deficient obese mice have lower levels of circulating free fatty acids, which are associated with the development of insulin resistance, and were protected from the obesity-related reduction in insulin receptor signalling in muscle and fat. This indicates TNF is an important mediator of insulin resistance and represent a potent target for insulin resistance in obesity.

1.9. TNF-α Blockers

Cytokines were first characterized in 1970s and TNF-α was cloned in 1984.73 The concept of anti-TNF-α therapy emerged around 1989 and the first clinical trial started in 1993, since then tremendous effort has been put to develop different types of TNF-α inhibitors (Fig.1.5.).
Fig. 1.5. Timeline showing development of anti TNF therapy for chronic inflammatory diseases.
1.9.1. Infliximab

Infliximab is a TNF-α blocking agent that is a chimeric immunoglobulins (Ig)G1κ monoclonal antibody composed of human constant and murine variable regions. It binds to both soluble and transmembrane forms of TNF-α with high affinity at picomolar concentrations. Binding to soluble TNF-α results in loss of bioactivity whereas binding to membrane-bound TNF-α leads to cytotoxicity by complement or antibody-dependent cell-mediated mechanisms. Evidence defining which mechanism(s) is relevant for infliximab is lacking.99,100

1.9.2. Etanercept

Etanercept is a dimeric fusion protein consisting of the extracellular ligand-binding portion of the human p75 TNF-α cellular receptor linked to the Fc portion of human IgG1. It is a recombinant product expressed in Chinese hamster ovary cells. The binding of etanercept with TNF-α is specific and interferes with TNF-α interaction with the cell surface TNF-α receptors p55 and p75. Inflammatory cells shed both TNF-Rs, forming soluble receptors that act as natural inhibitors of TNF-α. Therefore, the soluble TNF-Rs can be used as an alternative to the use of antibodies to neutralize TNF-α.

1.9.3. Adalimumab D2E7

Whereas infliximab is a monoclonal antibody consisting of a murine variable region and a human constant region, adalimumab neither is a fully human anti-TNF antibody, with neither nonhuman nor artificially fused human sequences, and it may, therefore, have lower immunogenicity. Early clinical trials with adalimumab showed similar responses to those seen for earlier trials with infliximab.101,93 This has completed Phase III trial recently, after a convincing Phase II trial.102

1.9.4. CDP-751

CDP-751 is a partially humanized anti-TNF-α monoclonal antibody with murine complementarity-determining regions grafted into a human IgG4. It has been tested and,
like infliximab, induced significant clinical improvements in patients with rheumatoid arthritis.\textsuperscript{94}

\textbf{I.9.5. Lenercept}

Lenercept is a TNF type I receptor Fc fusion protein which has also been used as monotherapy for rheumatoid arthritis and septic shock.\textsuperscript{103} Unlike entanercept, lenercept is immunogenic with repeated therapy and has been withdrawn from use because of this.

\textbf{I.9.6. PEGylated Recombinant Human Soluble TNF-R Type I}

The activities of TNF-\(\alpha\) are modulated by proteolytic shedding of the soluble extracellular domains of the two TNF receptors. A recombinant human soluble TNF-R type I (sTNF-RI) has been cloned and isolated by recombinant DNA techniques using \textit{E.coli} as host.\textsuperscript{104} A high molecular weight polyethylene glycol (PEG) molecule was attached at the N-terminus position to form the molecule intended for treatment (PEG sTNF-RI). It has been shown that PEGylation decreases both the rate of absorption and the plasma clearance of sTNF-RI which might have led to a more advantageous dosing schedule for patient compared with a non-PEG sTNF-RI.\textsuperscript{105} The administration of this drug to patients (clinical trial phase I and phase II), however, resulted in significant antibody production against the drug, which reduced its half life, clearance and eliminated its chance to be viable option for treatment.\textsuperscript{106}

\textbf{I.10. Targets for Therapeutic Interventions in RA}

\textbf{I.10.1. Inhibition of Cytokines}

TNF-\(\alpha\) and interleukin-1 are implicated in progression of arthritis. IL-1 acts in concert with TNF-\(\alpha\) to activate MMP-1 (collagenase) and MMP-3 (Stromlysin) in chondrocytes and fibroblasts like synovial cells. Moreover, IL-1 also inhibits the production of collagen and proteoglycans and stimulates bone re-sorption through the activation of osteoclasts.\textsuperscript{93,107,108} It has been postulated that TNF-\(\alpha\) drives most of IL-1 production by synovial tissue in RA. Therefore, blocking of TNF-\(\alpha\) is assumed to sufficiently down-
regulate all facets of the arthritis process. The first trial with IL-1 receptor antagonist (IL-1Ra) showed significant, but moderate, suppression of clinical disease activity. However, a beneficial effect on the rate of joint erosion progression was suggested in 6 month clinical trial.\textsuperscript{109,110} It is reported that IL-1 \(\alpha\beta\) blockade prevents cartilage and bone destruction in murine type II collagen-induced arthritis, whereas TNF-\(\alpha\) blockade only ameliorates joint inflammation.\textsuperscript{58} In addition to TNF-\(\alpha\) and IL-1, IL-6, IL-12 and probably also IL-15 and IL-18\textsuperscript{111-112,113} might prove to be valuable targets in chronic destructive arthritis.

\textbf{I.10.2. Adhesion Molecules}

In inflamed synovial tissue there is an increased expression of adhesion molecules, which probably increases the traffic of inflammatory cell into the joint tissue. Thus, a blockade of these molecules should lead to a reduced influx of inflammatory cells into the joint tissue as well as to a reduced inflammatory response in RA.\textsuperscript{114}

An open trial was performed employing an anti-intercellular adhesion molecule-1 (ICAM-1) MAb. In this study 10 patients with early RA were treated with anti-ICAM-1 MAb in a single 5-day infusion. Clinical improvements were seen in more than 50% of the patients.

\textbf{I.10.3. Inhibition of MMPs}

Matrix metalloproteinases (MMP) are enzymes that are involved in the destruction of joint surface and the repair process in RA pathogenesis.\textsuperscript{115} The process starts with activation of T cells that secrete proinflammatory cytokines, such as tumor necrosis factor alpha and interleukin-1, which in turn activate synovial macrophages and fibroblast to express MMPs, leading to tissue destruction.\textsuperscript{108,116} MMP inhibitors are currently being developed both by academic research groups and by industry. Several synthetic low-molecular-weight inhibitors of collagenase and gelatinase A and B have been examined for their inhibitory effects on bone resorption and type I collagenolysis.
I.11. Nucleic Acids as Tool for Therapeutic Interventions

Rational approach to drug development requires designing cognate molecules against chosen drug targets. This necessitates information on structures of the target and the rules governing its recognition by the drug. These rules are relatively simple in case of nucleic acids.

The concept of using RNA as therapeutic agents is relatively new, but has received increasing attention during past decade. Much of this interest stems from a variety of basic scientific discoveries that underscore the seminal role of RNA molecules in the utilization of genetic instructions in all living systems and the versatility of these molecules in nature. The therapeutic RNAs can be grouped as

I.11.1. Inhibition of Gene Expression

Regulation of gene expression by an RNA that is complementary to a target messenger RNA (mRNA) was first recognized as a naturally occurring process in prokaryotes.\textsuperscript{117}

I.11.1.1. Antisense Oligonucleotides

Antisense oligonucleotides are complementary to mRNAs and can specifically recognize their target transcript by forming base pairs with them in a sequence-specific manner. The formation of this RNA duplex is believed to lead to the degradation of the target RNA or the inhibition of translation. The ability to inhibit specific genes after gene transfer of antisense expression cassettes was first demonstrated almost two decades ago in bacteria\textsuperscript{118,119} and in eukaryotic cells.\textsuperscript{120} Later, numerous reports appeared that described the potential utility of antisense RNA for the inhibition of a wide array of genes in mammalian cells.\textsuperscript{121}

I.11.1.2. Ribozyme

In 1980s Thomas R Cech and Sidney Altman discovered that certain RNA molecules, dubbed ribozymes, could catalyze biochemical reactions that led to the development of a class of therapeutic RNAs called \textit{trans}-cleaving ribozymes. Such ribozymes bind
substrate RNA through base-pairing interactions, cleave the bound target RNA, release the cleavage products and are recycled so that they can repeat this process multiple times. The observation that such ribozymes can be repeatedly targeted to cleave virtually any pathogenic transcripts led to much speculation about their potential therapeutic value \textit{in-vivo}. This will be discussed in brief in next section.

1.11.1.3. siRNA

In 1998, Andrew Fire and Craig Mello described RNA interference (siRNA) that involves specific gene silencing by double stranded RNA (dsRNA). They showed that, in \textit{C. elegans}, the presence of just a few molecules of dsRNA was sufficient to almost completely abolish the expression of a gene that was homologous to the dsRNA. Since then different groups published vast number of articles demonstrating, siRNA mediated gene silencing in mammalian cells.\textsuperscript{122-125} RNAi is evolving at a faster pace than ever to use its potential in inhibiting the expression of pathogenic transcripts for therapeutic interventions.

1.11.2. RNA-Mediated Repair of Genetic Instructions

Genetic instructions are usually revised twice as they are converted from DNA to RNA to protein. Most of this revision occurs at the RNA level when splicing removes intron sequences from precursor transcripts and ligates together flanking exon sequences to generate mature RNAs. Several recent studies exploit this facet of RNA biology and describe the development of an intriguing new class of therapeutics RNAs that can perform \textit{trans}-splicing to repair clinically relevant mutant transcripts. Some of the initial studies showed that group I ribozyme could repair mutant \textit{lacZ} transcript in bacteria\textsuperscript{126} and mammalian cells.\textsuperscript{127}

1.11.3. RNA as a Protein Antagonist

Many small RNAs can fold into three-dimensional structures that allow them to bind target proteins with high affinity and specificity. Several RNA viruses such as HIV use this property of RNA to recruit viral and host proteins to perform essential functions in viral replication. The use of small-structured RNAs to directly bind and inhibit the
activity of pathogenic protein was first explored using the HIV (trans-activation response region) TAR sequence. Expression of TAR ‘decoy’ RNAs in CD4+ T cells was shown to competitively inhibit Tat binding to the viral TAR RNA and render cells highly resistant to HIV replication.128

I.12. Ribozymes

In 1980s Thomas R Cech and Sidney Altman discovered that certain RNA molecules, dubbed ribozymes, could catalyze biochemical reactions, a job previously thought to be the exclusive province of enzymes, which are proteins.129,130 Ribozymes are catalytic RNA molecules possessing the ability for specific cleavage of target RNA130-132 They function by binding with target RNA moiety through Watson-Crick base pairing and inactivate it by cleaving the phosphodiester backbone, this cleavage results in inactivation of the target mRNA. Since the initial landmark discovery of RNA with self-cleavage activity nearly two decades ago, several additional classes of ribozymes have been identified which are categorized on the basis of the reaction pathway or mechanism. Currently recognized groups are the group I intron, the group II intron, the RNA subunit of ribonuclease P, the hammerhead ribozyme, the hairpin ribozyme, and the hepatitis delta virus (HDV) ribozymes. The group I and II intron ribozymes and the ribonuclease P ribozyme are relatively large ribozymes (hundreds of nucleotides). The hammerhead, hairpin, and HDV ribozymes are substantially smaller (less than hundred nucleotides). The shortened form of the *Tetrahymena* group I intron is the mechanistically best characterized of the large ribozymes. Among the small ribozymes, the hammerhead is the best characterized and has been subjected to the most intense scrutiny, which has resulted in several X-ray structures.133-136

I.12.1. Hammerhead Ribozyme

The hammerhead ribozyme acquire hammerhead-like secondary structure that’s why they are called so. Fortunately this ribozyme is smaller in size and has been extensively studied. Hammerhead ribozymes were originally identified in analyses of plant viroids. They catalyze specific cleavage of RNA molecules131,132 and can cleave complementary substrate RNAs both in cis and trans. The hammerhead motif consists of three base-paired stems flanking a central core of 15 conserved nucleotides131,137 as
depicted in (Fig1.6). The conserved central bases, with few exceptions, are essential for ribozyme’s catalytic activity.

**1.12.2. Hammerhead Ribozyme Cleavage Reaction**

The *trans* acting hammerhead ribozyme consists of three helices of which two result from annealing with an antisense region (stem I &III) and the third belongs to a catalytic domain (Fig. 1.6). Hammerhead ribozyme has absolute requirement of divalent metal ions. Mg$^{2+}$ is the preferred metal, but Mn$^{2+}$, Co$^{2+}$, Sr$^{2+}$, and Ba$^{2+}$ also support cleavage activity.\(^{138}\) Standard reaction conditions include 10 mM metal, pH 7.5, at temperatures 27-50 °C. The cleavage reaction is initiated when the ribozyme binds to the substrate to form a Michaelis-Menten complex via formation of base pairs with stem I and III ($k_{\text{assoc}}$). Then a specific phosphodiester bond in the bound substrate is cleaved by the reaction of Mg$^{2+}$ ions ($k_{\text{cleav}}$). The cleavage generates products with 2', 3'-cyclic phosphate and 5'-hydroxyl groups. Finally, the cleaved fragments dissociate from the ribozyme and the liberated ribozyme is again available for new series of catalytic events. Individual rate constants for a complete kinetic scheme have been reported.\(^{139}\)

**1.12.3. Ribozyme Based Therapeutics**

Hammerhead ribozymes have been most extensively characterized in gene therapy applications. The requirement for hammerhead ribozyme mediated cleavage is presence of NUX site in target mRNA where N can be any nucleotides, U is uridine and X can be any nucleotides other than guanidine.\(^{140}\) In principle ribozymes can cleave any mRNA having NUX triplet. This gratifies ribozyme as potential therapeutic agents for inhibiting any pathogenic transcript in vitro.\(^{141-143}\) Numerous studies have shown the successful use of ribozymes for inhibition of gene expression in various organisms.\(^{132}\) Much progress has been made towards assessing the potential utility of *trans*-cleaving ribozyme, with the hammerhead and hairpin ribozyme\(^{137}\) being the main focus of this transnational efforts.

Ribozyme mediated regulation of gene in cell lines\(^{144,145}\) has been reported by many research groups. This provides the foundation of their potential therapeutic value *in-vivo*.\(^{146-148}\)
Fig. 1.6. Consensus sequence for the hammerhead ribozyme illustrated the three helical stem regions (I, II, and III) and the conserved, nominally single-stranded core sequence.
Several phase I and II clinical trials have been initiated using trans-cleaving ribozymes in a small number of patients with infectious diseases or cancer. In these studies the ribozymes have been delivered to the patients either by gene therapy methods or by direct injection of a synthetic ribozyme. The gene therapy-based trials have focused upon developing ribozyme-based treatments for individuals infected with the human immunodeficiency virus (HIV). Three separate groups have used retroviral vectors to introduce expression cassettes for anti-HIV ribozyme into CD4+ lymphocytes or CD34+ haematopoietic precursors ex vivo that have been taken from the infected patient or from an identical twin.\textsuperscript{149-151} The transduced cells are then infused into the patient and the engraftment and survival of the ribozyme-cotaining cells are monitored.

Initial results from these studies suggest that transfer of ribozyme-encoding genes to HIV-infected individuals is well tolerated and transduced cells can persist in the patient.\textsuperscript{150} Moreover, preliminary reports suggest that anti-HIV ribozyme-containing cells may possess a transient survival advantage in the patient compared with cells transduced with a control vector. Different research groups have targeted the genes implicated in pathogenesis of arthritis. There are some studies showing the in vitro and ex vivo cleavage of murine TNF-α mRNA with cognate ribozyme.\textsuperscript{152,153}

Expression of hammerhead ribozyme against human tumor necrosis factor alpha (hTNF-α) caused a decrease of the titer of apoptotic factor in culture supernatants of THP1 cell.\textsuperscript{154}
I.13. Aim of the Present Study

TNF-α is a prime target for therapeutic interventions in rheumatoid arthritis. The main objective of the present research was (a) to study the efficacy of TNF-α targeting ribozyme to inhibit TNF-α production cell line in-vitro, and (b) to study its therapeutic potential in ameliorating collagen induced arthritis in DBA/1 mouse. The study involves the following work elements:

- Designing of Ribozyme against murine TNF-α.
- Cloning of cDNA for murine TNF-α.
- Evaluation of the cleavage efficiency of designed ribozyme, in vitro.
- Evaluation of factors (Glucose-6-phosphotase, Mg^{2+} and Ca^{2+}) affecting ribozyme-mediated catalysis in vitro.
- Evaluation of ribozyme expression in murine macrophage cell (J774A.1) line.
- Ribozyme mediated depletion of murine TNF-α in J774A.1 cell line.
- Expression of TNF-α targeted ribozyme in DBA/1 mice.
- Induction of collagen induced arthritis (CIA) in DBA/1 mice.
- Effect of TNF-α targeting ribozyme on CIA mice.
  a) Early ribozyme treatment on day 8\textsuperscript{th} after BCII immunization.
  b) Treatment during the period of arthritis onset, day 18\textsuperscript{th} after BCII immunization.
  c) Treatment after appearance of clinical symptoms.
  d) Monitoring the disease in terms of physical observation.

- Histology for joint pathology score.
- Anti-BCII antibody quantitation in serum of treated mice.
- Inflammatory cytokines quantitation in serum of treated mice.