Chapter 2:

Review of Literature

2.1. INFLAMMATION

Inflammation is the immediate manifestation of defense by the immune system against any foreign substance that has entered the body. The inflammation is generally a quick response initiated against any foreign particle which is characterized by swelling, redness, heat and pain (Firestein, 2003). According to Medzhitov (2008), inflammation is a biological reaction to a disrupted or disturbed tissue homeostasis. At its basic level, it is a tissue-destruction process which involves the recruitment of blood-derived products and blood cells such as plasma proteins, plasma fluid, and leukocytes into perturbed tissue. Inflammation is a pervasive defense mechanism that is broadly defined as a nonspecific response to tissue malfunction and is employed by both innate and adaptive immune systems to combat intruding pathogens. Unlike the other defense mechanisms of the immune system, partial damage of the host is unavoidable in inflammation (Ashley *et al*., 2012). Interactions of the cells and components of the innate immune system, adaptive immune system, and inflammatory mediators orchestrate the mechanisms and aspects of the inflammation that underlie different diseases in different organs. Combination of common effector mechanisms of inflammation contribute to tissue injury, oxidative stress, extracellular matrix remodeling, angiogenesis, and fibrosis in diverse target tissues (Libby, 2007). Inflammation-induced collateral damage in the host body might contribute to immunopathology [for example, diseases like rheumatoid arthritis (RA), multiple sclerosis (MS), diabetes etc] but the damage invoked by inflammation represents the biological balance mechanism between the damage control against incoming pathogens and self-maintenance; the process initiates with the identification of invading pathogen and terminates as the invading pathogen is neutralized. It does not require the presence of self-antigens to become activated in most of the cases (Ashley *et al*., 2012). However, a delay in the termination of adaptive immune response leading to the prolonged inflammation often directs to autoimmune diseases. It involves both genetic and environmental factors and mechanisms including the lack of self-antigen recognition (Graham *et al*., 2005).

Inflammation can be classified into two different types depending on their duration. Acute inflammation is the immediate type of response which lasts for limited period of time, usually for a few hours to few days. A more prolonged type is called the chronic

inflammation which shows a longer duration for termination. Another classification has been demonstrated depending on of the intensity of the inflammatory reaction. These are low-grade and high-grade inflammations (**Table 2.1**) (Ashley *et al*., 2012).

Table 2.1. Types of inflammatory responses categorized by duration (low-grade and high-grade) and degree of intensity (acute and chronic) (Modified from Ashley *et al*., 2012).

Based on duration **Based on intensity**	**Acute inflammation**	**Chronic inflammation**
Low-grade inflammation	Para-inflammation, Metaplasia	Inflammatory diseases, Autoimmune disorders, Neurodegenerative diseases, Tumour growth, Tissue damage and fibrosis
High-grade inflammation	Acute phase response, Release of cytokines, Neutrophil migration, Recruitment of effector cells, Localized tissue damage	Sepsis, Cytokine storm, Tissue destruction

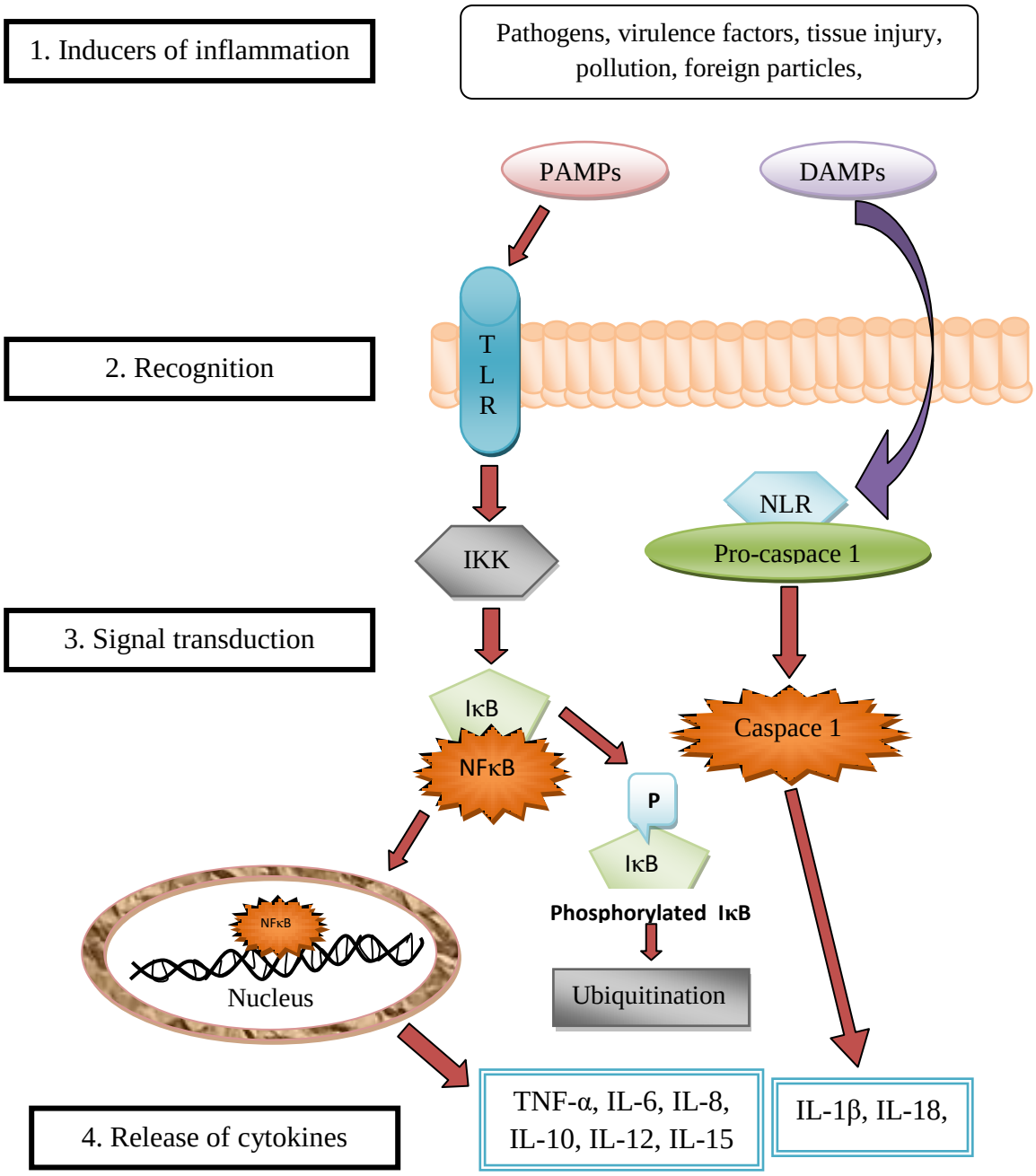
2.1.1. Mechanism of Inflammation

Pathogens, tissue injury, and foreign particles induce inflammation in the host body. Transmembrane TLRs (Toll-like receptors) and intracellular NLRs (nucleotide binding domain and leucine-rich-repeat-containing receptors) bind to PAMPs (pathogen associated molecular patterns) or Alarmin like DAMPs (damage-associated molecular pattern) of the incoming particles, respectively (Lange *et al*., 2001). The recognition of non-self-molecular patterns by the host cell receptors then initiate signaling pathways in the cell for a response. TLRs activate a MyD88-dependent signal transduction pathway that phosphorylates the inhibitory IκB protein by IKK (inhibitor of kappa B kinase). As a result, NF-κB is released from phosphorylated IκB, and then it translocates to the nucleus where it up-regulates transcription of different inflammatory genes (Ghosh *et al*., 1998). These genes include pro-inflammatory cytokines like TNF-α, IL-2, IL-6, IL-8, IL-10, IL-12, IL-15 etc. On the other

hand, NLRs convert procaspase-1 into activated caspase-1. The caspase-1 functions in the conversion of different cytokines into active forms (IL-1β, IL-18 etc). All these cytokines participate in inflammation. A variety of pro-inflammatory cytokines and chemokines which are produced and released at the site of inflammation by the inflamed cells as well as by the immune cells promote effector functions of inflammation. At the same time, blood-borne neutrophils and monocytes also migrate to the site of inflammation by chemotaxis passing through the endothelial cells, a process known as extravasation. This influx of cells is also associated with the accumulation of protein-rich fluid, known as the 'exudate' promoting edema at the site of inflammation (swelling). Mast cells and tissue-resident macrophages help in this migration of cells by releasing histamine, leukotrienes, and prostaglandins, which have rapid effects including vasodilation of the blood vessels and increased vascular permeability.

Vasodilatory prostaglandins play an important role in the manifestation of inflammation, where Cox-2 plays a role as a key molecule and contribute to the increase in the degree of inflammation (Sherwood and Toliver-Kinsky, 2004). On the other hand, neutrophils release toxic compounds, including reactive-oxygen species (ROS), reactive-nitrogen species (RNS), and various proteases, which are nonspecific and harmful to both the pathogen and to the host. Macrophages and dendritic cells participate in phagocytosis of cellular or particulate antigens as well as the cellular debris at the inflamed region (Nathan 2002; Ashley, 2012). In case of prolonged inflammation, the antigen presenting cells (APCs) like dendritic cells (DC), marcophages (Mφ), mast cells etc also migrate to lymphoid tissue and prime naive T-cells (Th_0) to become polarized through stimulation of the T cell receptor (TCR). Th_0 cells differentiate into several different types of effector and regulatory T-cells including Th1 cells, Th2 cells, $T_{reg,}$ and Th17 cells. Th_1 cells generally secrete pro-inflammatory cytokines; Th2 cells are responsible for of B-cell activation, whereas Th17 cells are highly pro-inflammatory and are regulated by the other Th subsets. T_{reg} cells contribute to the degree of inflammation (Abbas *et al*., 1996). The resolution of inflammation occurs as the suppression of neutrophil recruitment at the inflamed site, triggered by lipoxin A4 produced by marcophages; apoptosis of inflamed cells; and clearance of the cellular debris and mediators formed during inflammatory responses by the phagocytic immune cells. Also, the decrease in the titre of immunologic molecules, depending on their half-life,

contributes to the termination of inflammation. Fas ligand, resolvins, and protectin molecules promote the apoptosis of neutrophils. Macrophages and dendritic cells phagocytose apoptotic neutrophils and cellular debris. Termination of inflammation is crucial for limiting the collateral damage to the host body (Serhan and Savill, 2005; Ashley *et al*., 2012). The process of inflammation can be categorized into following events: (1) introduction of the inducers of inflammation, (2) inducer recognition by the host, (3) signal transduction in the host cell, (4) release of pro-inflammatory cytokines, (5) effects of inflammation, (6) polarization of inflammation, and (7) resolution of inflammation. The process of inflammation is summarized in the following figure (**Fig. 2.1**).


1. Inducers of inflammation
Pathogens, virulence factors, tissue injury, pollution, foreign particles,
PAMPs
DAMPs
2. Recognition
T
L
R
NLR
IKK
Pro-caspace 1
3. Signal transduction
IκB
NFκB
Caspace 1
P
IκB
Phosphorylated IκB
Ubiquitination
NFκB
Nucleus
4. Release of cytokines
TNF-α, IL-6, IL-8, IL-10, IL-12, IL-15
IL-1β, IL-18,

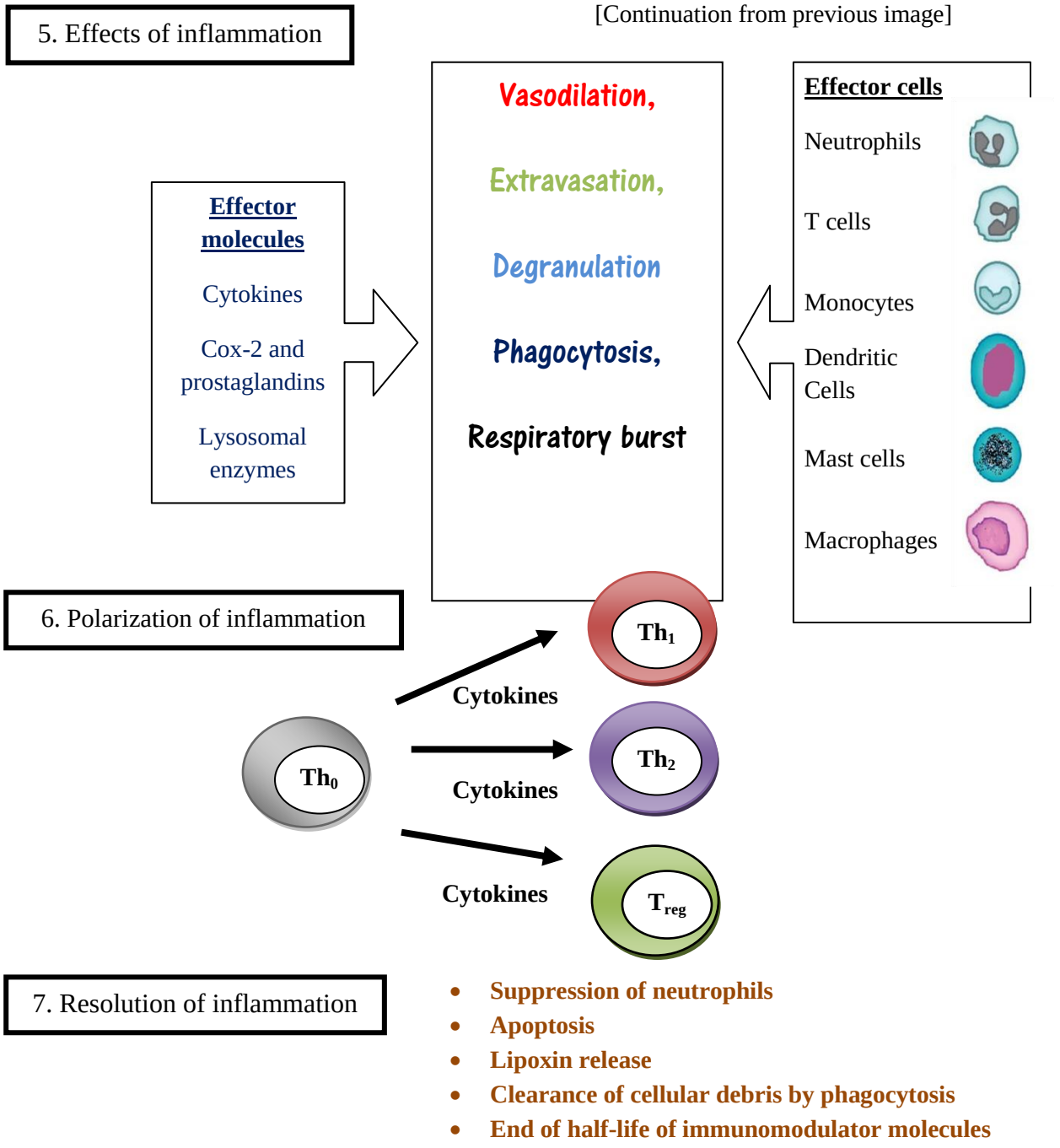


Figure 2.1. Key events of inflammation (mentioned as 1-7 on the left side) and important mediators of the progression of inflammation (Steps 1-7 on the right side). Modified from Ghosh *et al*. (1998), Janeway *et al*. (2005), Anthony *et al*. (2007), Soehnlein and Lindbom (2010), Maskrey *et al*., (2011), Ashley *et al*., (2012). IκB: nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor; IKK: inhibitor of kappa B kinase; IL: Interleukin; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; Th: T helper cell; TNF-α: tumour necrosis factor-alpha; Treg: regulatory T cell.

2.2. RHEUMATOID ARTHRITIS (RA)

Among the different inflammatory diseases, Rheumatoid Arthritis (RA) is one of the major chronic inflammatory diseases found world-wide. RA is a chronic systemic inflammatory auto-immune disorder seen in the bone joints of human. It has shown a worldwide prevalence of approximately 0.5% to 1% among adult individuals (Scott *et al*., 2010). It differs from other types of arthritis as it is auto-immune, where the immune system of the patient's body reacts against the self or altered-self antigens produced within the body.

2.2.1. General characters and epidemiology of RA

The disease is externally identified by the swelling and redness of a joint present for an extended period of time (at least for more than 6 weeks). The clinical characters are synovial hyperplasia, inflammatory cell infiltration in the synovial tissues, destruction of cartilages and bones in joint region all leading to chronic disability (McInnes and Schett, 2011). Bone joint destruction is associated with severe chronic inflammation which is associated with the damage to the surface and extracellular articular cartilage matrix and degradation of bones. If the situation remains undiagnosed, it hampers the quality of lifestyle. Physical movements is highly disturbed in severe diseased condition leading to partial or complete immovability of the patient.

Depending on different population-based studies, RA has shown a global prevalence of approximately 0.5% to 1% among adults (Scott *et al*., 2010). According to the Global Burden of Disease Study report (Roth *et al*., 2018), the prevalence rate of RA is about 0.24% among the world population (Cross *et al*., 2014) and females have twice the more chances of having the disease than that of the males (van Vollenhoven, 2009). The prevalence rate of RA is much higher in the Australian region followed by North America and Europe; however, shows a less prevalence in the Asiatic region (Gonzalez *et al*., 2007; Cross *et al*., 2014). It is hypothesized that, one in twelve females and one in twenty men would develop an inflammatory autoimmune rheumatic disease during their lifetime (Crowson, 2011). In the developing countries and low and middle income countries like India, however, the actual number of patients suffering from RA could be higher than the available data because of the

lack of extensive survey and the inadequate statistics regarding the number of patients from deep rural regions (Rudan *et al.*, 2015).

2.2.2. Classification criteria for RA detection

To establish the epidemiology of a disease, a detailed population-based study has been an integral part. It provides important information to assess disease possibility and it is the primary tool in disease detection. In the last 50 years, in the field of rheumatic diseases, newer approaches have come and have changed or modified the classification criteria for rheumatic diseases. Felson and Anderson (1995) defined a number of steps for the development of a classification criterion for rheumatic diseases. However, the first approach to understand the classification criteria for RA was done by the American Rheumatism Association in 1956 (Hochberg, 2009) and it was revised and widely accepted in 1958 based on 332 cases and controls from 19 different cities across US and Canada. It included 11 symptoms or characters and their possible combinations which gave rise to four disease phases based on severity (**Table 2.2**).

The '1958 American Rheumatism Association's (ARA) criteria' was further modified in '1987 American College of Rheumatology (ACR) criteria'. In this period of time, different seronegative arthritic conditions were discovered and a modification was required. The newer format of 1987 came out with both 'tree' and 'list' format. The tree format was more appropriate to detect the presence of disease in patient body (**Table 2.3; Fig. 2.2**).

Table 2.2: 1958 American Rheumatism Association criteria for RA. (Modified from Hochberg, 2009)	
1.	Morning stiffness
2.	Pain on motion or tenderness of at least one joint
3.	Swelling in at least one joint
4.	Swelling of at least one other joint within the span of three months from the swelling of first joint
5.	Symmetrical joint swelling with simultaneous involvement of the same joint on both sides of the body
6.	Subcutaneous nodules over bony prominences
7.	X-ray changes typical of RA including decalcified bones around the involved joint
8.	Positive agglutination test for rheumatoid factors
9.	Poor mucin clot from synovial fluid
10.	Characteristic histologic changes in synovial membrane including infiltration of inflammatory cells, marked villous hypertrophy, deposition of compact fibrin, foci of cell necrosis
11.	Characteristic histological changes in nodules including necrosis zones, inflammatory cell infiltrations.
Types of RA based on 11 criteria	
Classical RA	At least 7 criteria must be present. For criteria 1 to 5, symptoms must be present for at least 6 weeks.
Definite RA	At least 5 criteria must be present. For criteria 1 to 5, symptoms must be present for at least 6 weeks.
Probably RA	At least 3 criteria must be present. For criteria 1 to 5, symptoms must be present for at least 6 weeks.

Table 2.3: Revised criteria for determination of RA by American Rheumatism Association in 1987 (Modified from Hochberg, 2009).		
1.	**Morning stiffness**	Morning stiffness in and around the joints, lasting at least 1 hour before maximal improvement.
2.	**Arthritis in 3 or more joint areas**	Soft tissue swelling of fluid accumulation (not bony overgrowth) present at least for 6 weeks
3.	**Arthritis in hand joints**	Swelling of wrist metacarpophalangeal joints (MCP) or proximal interphalangeal joint (*PIP*) for at least 6 weeks
4.	**Symmetrical arthritis**	Simultaneous involvement of same joint areas on both sides of the body (exception for symmetrical arthritis are the joints listed in point 3)

5.	**Rheumatoid nodules**	Subcutaneous nodules over bones or extensor surfaces or in juxta-articular regions
6.	**Rheumatoid factor**	Detected by a method positive in less than 5% normal controls
7.	**Radiographic changes**	Typical RA radiographs of joints with bone decalcification localized or adjacent to the involved joints

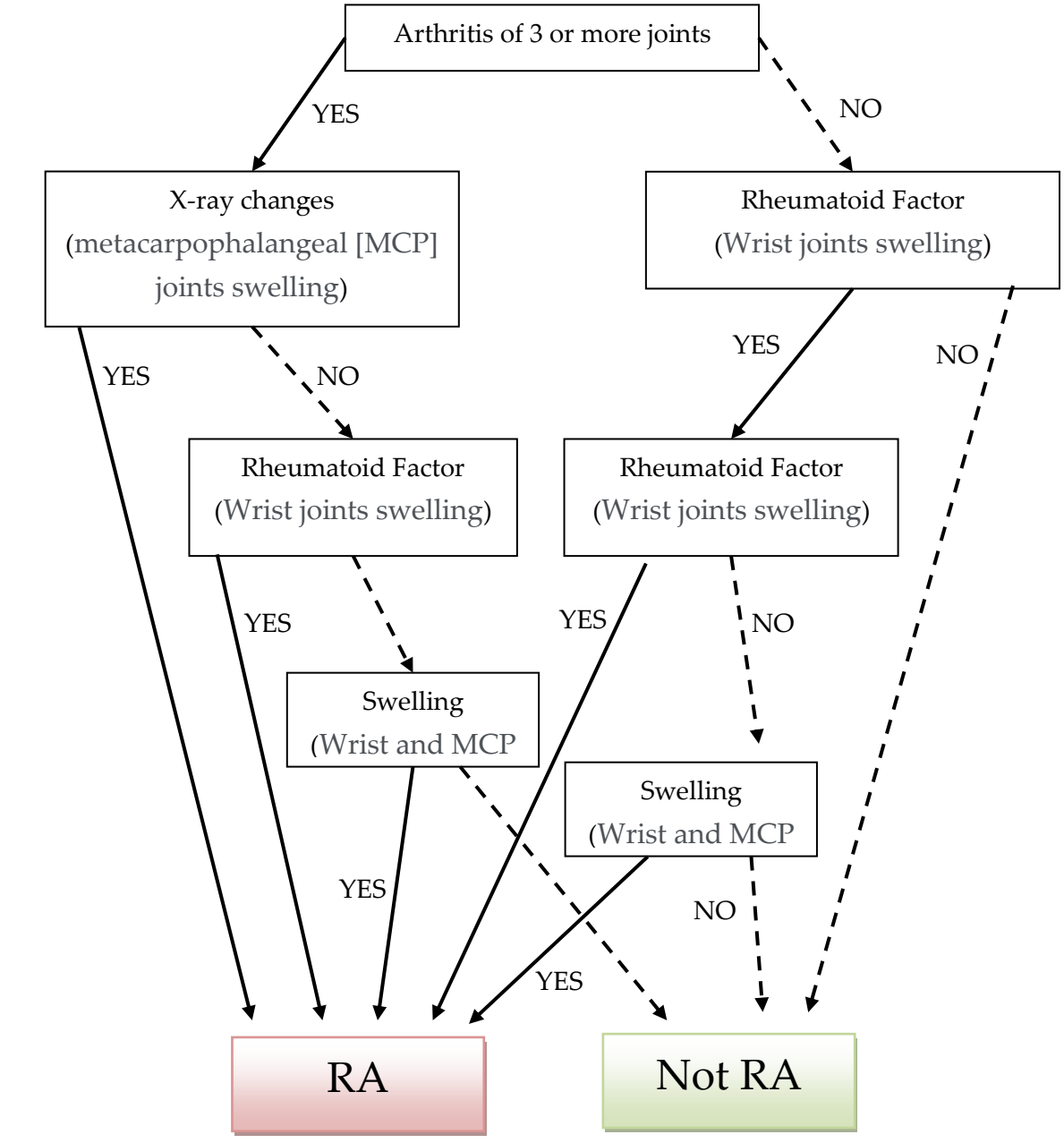


Figure 2.2. Decision-tree developed by American Rheumatism Association in 1987 for the detection of RA (the 'tree format'). (Modified from Hochberg, 2009).

However, the 1958 ARA criteria have been used for approximately 30 years and then it was revised in 1987 which is also over 30 years old. The detection of inflammatory polyarthritis and a few more complicated variants of arthritis need a better 'biomarker signature' which will combine with the both the genetic and serological status, advanced imaging techniques and cytokine profiles (Hochberg *et al*., 2009).

2.2.3. Risk factors of RA

Rheumatoid arthritis is regarded as a multi-factorial autoimmune disorder. Risk factors for rheumatoid arthritis comprise a combination of genetic, environmental and stochastic factors. Twin studies have estimated that the genetic risk factors count for almost 50% in the RA and the environmental and stochastic factors counts for the rest (Aho *et al*., 1986; Silman *et al*., 1993; MacGregor *et al*., 2000) (**Fig. 2.3**). RA is also characterized by the presence of auto-antibodies and auto-reactive T-cells in blood and joint fluid which are missing in case of the other join degenerative inflammatory diseases like psoriatic arthritis, reactive arthritis, osteoarthritis etc. Citrullinated proteins are formed in the host body as a result conversion of arginine into citrulline (Nienhuis and Mandema, 1964; Girbal-Neuhauser *et al*., 1999).. As the citrulline is not one of the essential amino acids, these citrullinated proteins are considered as foreign and are attacked by the immune system. There is a high association of citrullinated protein antigens and the chances of RA (Coenen *et al*., 2007). RA can be divided into two distinct subsets depending on the presence/absence of anti-citrullinated protein or peptide (auto-) antibodies (ACPAs). The major genetic factors are generally pre-deposited in the patients of ACPA-positive rheumatoid arthritis includes HLA-DR alleles, PTPN22 risk alleles, TRAF1/C5-related genes (Schellekens, 1998; Schellekens, 2000). IRF-5 is a generic factor associated with ACPA-negative RA. Smoking is strongly associated with ACPA-positive and RF-positive RA which is an environmental factor introduced through life-style choice. The different risk factors can overlap and different combinations of these factors can be observed in patients suffering with RA. RA has a polygenic basis, which means more than one and unrelated genes are involved in the disease and 31 different risk loci including HLA-SE alleles, PTPN22, C5-TRAF1, CTLA4, STAT4, HLA-DR3, IRF5 etc have been identified (Stahl *et al*., 2010). The presence of these loci in

different combinations increases the chance of the disease occurrence by several folds (Stahl *et al*., 2010).

The environmental risk factors determined through epidemiological studies includes silica-dust exposure (Klockars *et al*., 1987), smoking (Vessey *et al*., 1987), mineral oil and adjuvants (Sverdrup *et al*., 2005) etc. The stochastic factors include blood transfusion, dietary factors, obesity, social class and sports injury (Hochberg, 2009).

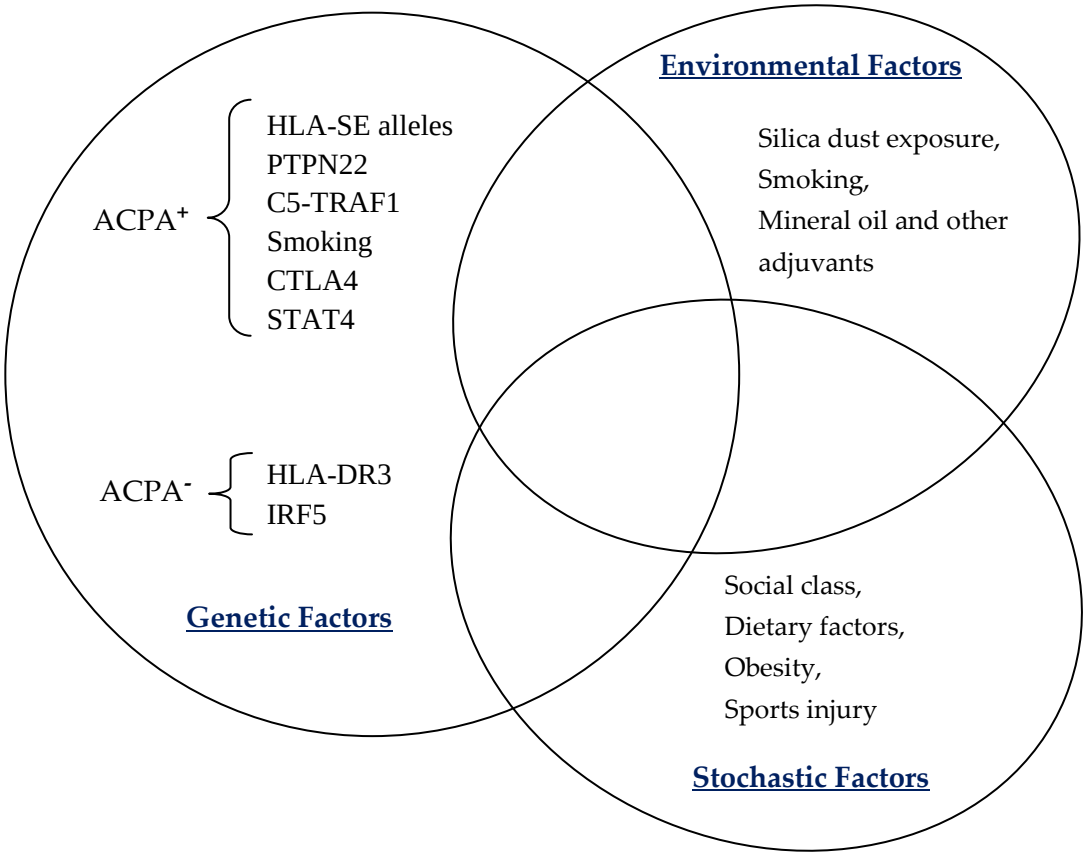


Figure 2.3. Different risk factors of RA. In different RA cases, several factors overlap and contribute to the multi-factorial nature of the disease. (Modified from Hochberg, 2009)

2.2.4. Pathogenesis of RA

Initiation of the disease: Generally the rheumatoid arthritis and other arthritic diseases show a few years span for their development and the progression continues for the rest of the life once the disease is initiated. In patients it can last up to a few decades. However, the progression of the disease can be divided into two distinct time-frames depending on its first

appearance and its duration. From the time of appearance of initial symptoms like joint inflammation, joint swelling, morning stiffness and symmetrical appearance of joint pains, a time-frame of several weeks or even a few months can be considered as 'early arthritis' stage. This stage has also been named as the 'window of opportunity'. This opportunity window suggests that the intervention of drugs like DMARDs in this phase could delay or down-regulate the progression and severity of the disease. The cytokine pattern and disease state at this period is different from those of the later disease phases (Raza, 2019). However, the duration of this 'early stage' and onset of the 'later' chronic stage of the diseases remains doubtful as there are no fixed criteria and time frame for the two phases and the durations vary from patient to patient.

As mentioned earlier, the disease has some strong association with several types of auto-antibodies, including rheumatoid factor (RF), anti-perinuclear factor (APF) and anti-keratin antibodies (AKA), anti-collagen antibodies, nuclear antigen-associated antibodies like Epstein-Barr nuclear antigen, RA33, anti-citrullinated vimentin or anti-Sa and anti-p68 antibodies (Smolen and Steiner, 2001). It has already been stated that most of these antibodies react or interact with citrullinated proteins (Schellekens *et al*., 1998). But it is still unknown whether such auto-antigens initiate the T-cell activation cascade from the very beginning to form inflammatory changes, or contribute at a later stage of the disease progression to flare up the disease.

Morphological changes: RA is an autoimmune, inflammatory disease. The symptoms can be identified by pain, swelling, stiffness at different joints in the patient body. Swelling of the joints associated with severe pain is the most visible feature of the disease from the initial period of the disease progression. These are also considered to be the initial criteria for the diagnosis of the disease. When the disease progresses in the patient's body, gradually the loss of function of the joints (most commonly in the hands, wrists, and knees) and joint deformities leads to structural and functional unsteadiness of the joints as well as of the whole body. Deformation of bones leads to permanent shape change of the body parts (CDC Guideline, 2020). However, many morphological changes are not specific to the rheumatoid arthritis only; many other arthritis including osteoarthritis includes the similar symptoms. There is generally a bilateral/symmetrical pattern of disease progression (e.g., both hands and

both knees are affected). The other associated changes in the patient's body include fatigue, fever, and loss of appetite.

Histological and radiographic changes: A vast array of histologic changes appears in the synovial membrane of rheumatoid arthritic joints. These include the proliferation of the synovial lining, lymphocyte and plasma cell infiltration, increased fibrosis and vascularity (Rooney *et al*., 1988). The immune cells appear in the arthritic synovium as a part of the immune defense mechanism against the self-antigens. The aggregation of lymphocytes in the sub-chondral side of the joint in rheumatoid arthritis is often associated with the presence of osteoclasts (McQueen and Ostendorf, 2006). In a study based on the bone samples of RA patients, it was clearly visible that increased number of osteoclasts adherent to the sub-chondral bones and lymphoid neogenetic feature of the bone marrow were associated with the progression of the disease (Bugatti *et al*., 2005). The simplified diagrammatic presentation of a healthy joint and arthritic bone joint has been shown in **Fig. 2.4**. There are evidences that the RA synovium shows degrees of variation among patients. The synovial lining in the inflamed joint seems to have fewer cell layers compared to the healthy joint. Diffused lymphocyte infiltration was observed all around the synovium and partial fibrinoid necrosis is observed (Tsubaki *et al*, 2005). In the radiographic imaging obtained from patients, visible joint destruction is observed in 70% of the patients within their first 2 years of disease. Radiographically visible damage of the bone joints appears rapidly over the first few years. It is followed by a progressive increase of joint erosion for the successive 20 years throughout the lifespan. The hallmarks of RA of radiography include symmetric alignment abnormalities of the joints, periarticular osteoporosis, joint space narrowing, sometimes rheumatoid odules and synovial cysts. Other imaging systems like computed tomography (CT) and magnetic resonance imaging (MRI) also provide excellent visual cues to identify arthritis.

Hematological and serum profile changes: Along with the prominent morphological changes, the infliction of RA can be determined through the assessment of changes in the serum properties of the patient as well as from hematological changes. A nonspecific decrease in hemoglobin level (up to less than 10 g/dl) and RBC along with the increase in lymphocytes and WBCs are characteristic features of RA presence. The C-reactive proteins

are generally increased which is associated with an elevation in erythrocyte sedimentation rate (ESR). Increase in the concentration of serum cryoglobulins and participating antibodies is also evident (Epstein 1990). Conditions like eosinophilia (Winchester *et al*., 1971) and thrombocytosis (Hutchinson *et al*., 1976) are also associated with the progression of the RA. Active RA is also associated with anemia of chronic disease and lymphadenopathy, and there is also an increased risk of non-Hodgkin lymphoma compared to the general population (Hochberg, 2009).

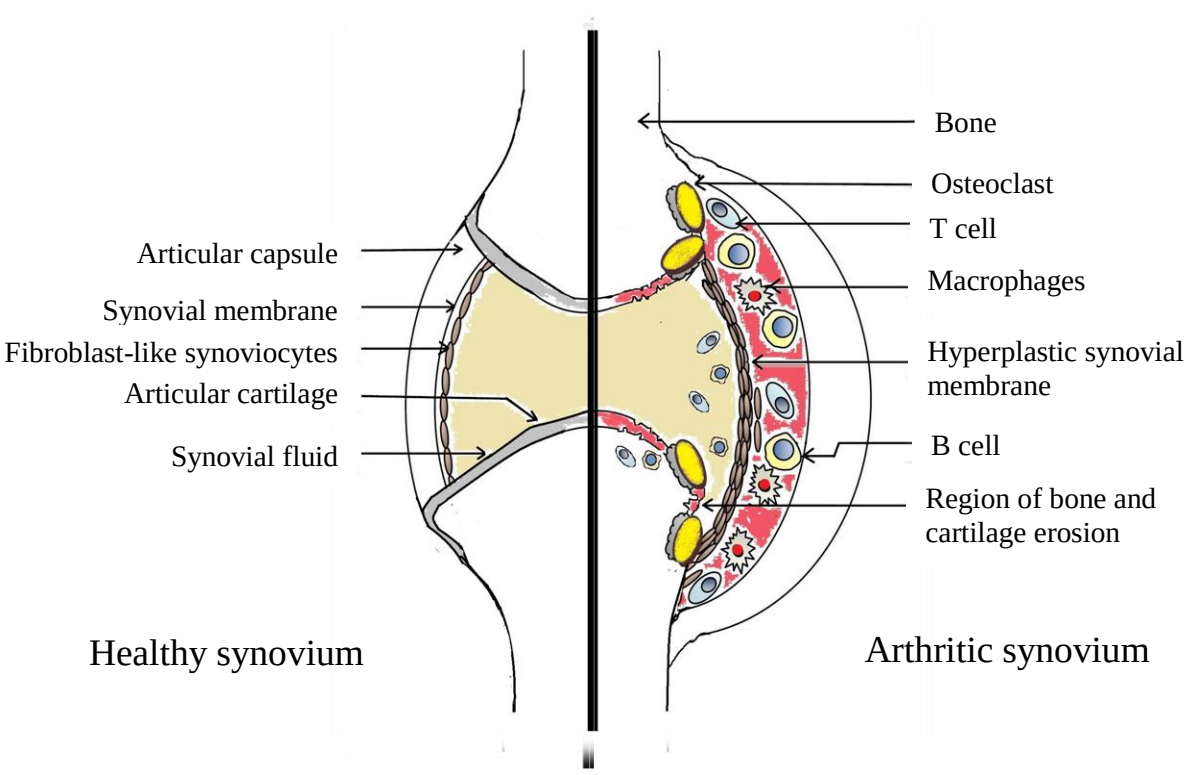


Figure 2.4. Diagramatic presentation of a healthy synovium (left-hand side) and an arthritic synovium (right-hand side). In the arthritic condition, the synovium and the joint cartilage is infiltrated by different immune cells including T cell, B cell and marcophages resulting in synovium degradation and cartilage erosion.

Interaction of innate and adaptive immunity in the disease progression: The innate immune response in the genetically pre-disposed individuals is possibly activated by events such as the triggering of dendritic cells (DCs) through Toll-like receptors (TLRs). TLRs are expressed on different cell types of the body including synovial cells membranes. These

TLRs can bind and recognize exogenous material or foreign stimuli together with autologous antigens (Thomas *et al*., 1999; Firestein 2003; Cavanagh *et al*., 2005). These recognitions initiate an inflammatory process within the host cells which has been discussed earlier. As the inflammation continues, synovial dendritic cells activated by TLR ligands migrate to lymph nodes and initiate the polarization of inflammation. In the lymph nodes, activation of primed T-cells is done which are biased towards the Th1 phenotype. The synovial membrane is then infiltrated by T-cells, which produce IL-2 and IFN-γ. So the T-cell response attains a Th1 bias. Th1 cells also trigger delayed type of hypersensitivity (Abbas *et al*., 1996). When first stimulated with antigens and APCs, naive $CD4^+$ T-cells also secrete low amount of IL-4, which acts for Th2 differentiation. Several other cell types are also reported to secrete IL-4 including mast cells, basophils, NK cells, and autocrine Th2 cells themselves (Lafaille, 1998). These Th2 cells help the activation of B-cells for the production of immunoglobulins (Ig) such as IgG1 and IgE antibodies during the progression of the disease and induce type I hypersensitivity (Abbas *et al*., 1996). The interaction of different APCs, T-cells, B-cells and their effector molecules is summarized in **Fig. 2.5**. Further, Th17 cells, which are the IL-17 producing subsets of Th cells, are also an important effector T-cell subset in RA development and progression. It induces the release of a wide range of pro-inflammatory molecules including IL-6, prostaglandin E2, IL-1β, TNF-α etc (Kinne *et al*., 2000; Cope 2008). The role of IL-17 has been confirmed in the pathogenesis and development of RA-like conditions in the experimental animals (Lubberts *et al*., 2002), however, the role of Th17 cell types in human arthritic synovium is less clear (Kotake *et al*., 1999).

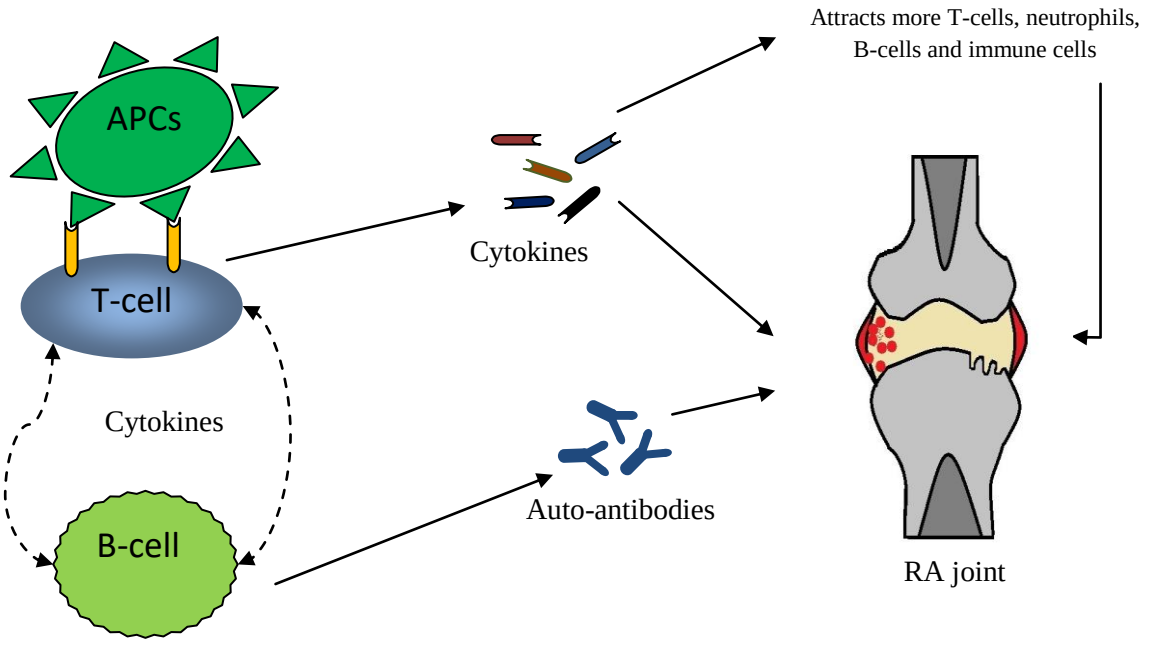


Figure 2.5. Interaction of antigen presenting cells (APCs; dendritic cells, macrophages etc), T-cells, B-cells and cytokines in the progression of RA in the synovium (Modified from Hochberg, 2009).

The direct and indirect role of B-cells and its auto-antibodies and/or immune complexes plays important role in the propagation and enhancement of the inflammatory process of RA. B-cells function as effective antigen presenting cells (APCs) and also produce the auto-antibodies against RF (IgM-RF) and citrullinated proteins (Anti-CCP IgG Antibodies) in 60-80% of the patients (Silverman and Carson, 2003). Immunoglobulin production in synovium also contributes to the local formation of immune complexes and activation of coagulation system. B cells also produce pro-inflammatory cytokines which up-regulates the activation of macrophages, antigen-presenting dendritic cells, regulates differentiation of follicular dendritic cells (Dorner and Burmester 2003). The different roles played by the B-cell in the progression of RA have been shown in the **Fig. 2.6**.

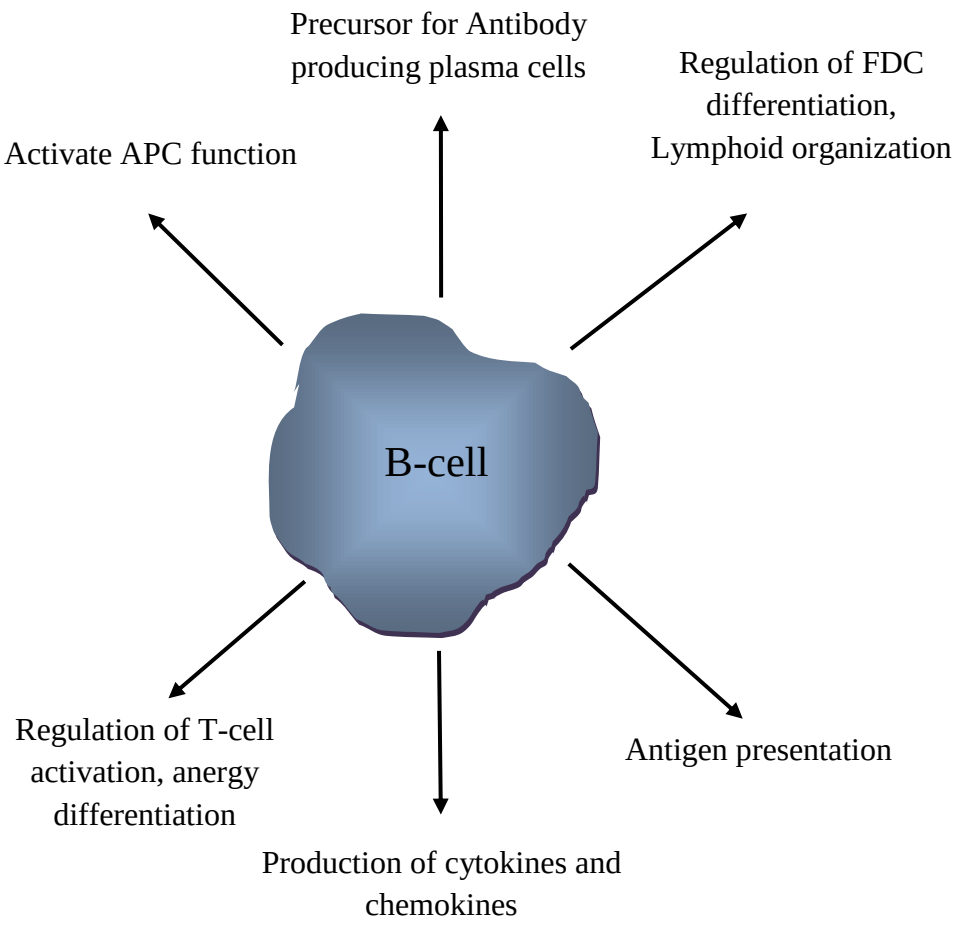


Figure 2.6. The various functions of B-cells. APC, antigen-presenting cell; FDC, follicular dendritic cell during RA progression (Modified from Hochberg, 2009).

Role of cytokines in RA progression: The most important regulators of the progression of inflammatory RA are the cytokines. They control the broad systemic effectors of RA in patients. The wider effects of cytokines have been proved in animal model-based experiments. Experimental evidences suggest that antigen-induced arthritic murine models lacking beta-chemokine 5 and 7 (CCR5 and CCR7) receptors show reduced inflammatory parameters in experimental condition. CCR5 and CCR7 specifically bind and respond to cytokines of the CC chemokine family. Animals lacking the CCR5 and CCR7 receptors also show inhibition of ectopic germinal-centre formation during inflammation (Wengner, 2007). On the other hand, in the proof-of-concept clinical studies, where cytokine blockers has been introduced into animal models or patients, has been successful in the assessment of potential role of different cytokines including TNF in the progression of RA (Maki-petaja *et al*., 2006).

Several groups of researchers have worked on the cytokine mRNA expression in the inflamed synovium of RA patients. Despite the differences in the duration of the disease, disease severity and treatment remedies between patients, all RA operative samples and biopsies showed the over expression a broad spectrum of pro-inflammatory cytokines. These results indicated that TNF-α is an apex pro-inflammatory cytokine. Some other major pro-inflammatory cytokines identified are IL-1, IL-2, IL-10 etc. Analogous studies also evaluated the expression of anti-inflammatory cytokines like IL-6, IL-8 GM-CSF etc in the synovium of RA patients. With the progression of the inflammatory disease, rheumatoid synovium can be envisaged as an equilibrium, becoming tilted towards the pro-inflammatory side (Feldmann *et al*., 1996). The brief idea of the involvement of different cytokines has been described in the **Fig. 2.7**.

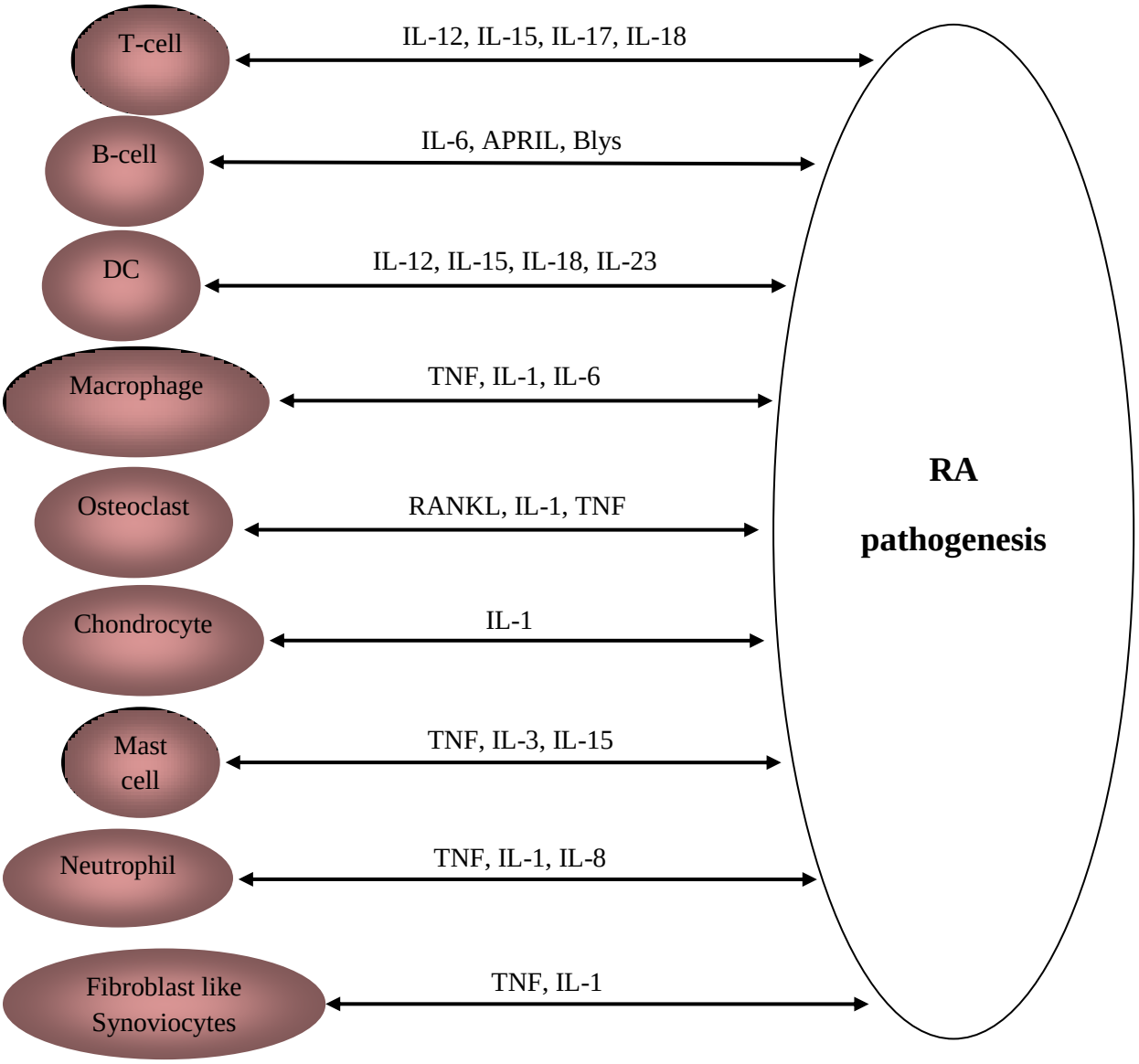


Figure 2.7. A notional idea about the effects of key cytokines in the progression of RA through the modulation of different immune cell types. (Modified from Hochberg, 2009) APRIL: a proliferation-inducing ligand; BLyS: B lymphocyte stimulator; RANKL: Receptor activator of nuclear factor kappa-B ligand.

The aggregation of immune cells in the synovium overproduce pro-inflammatory cytokines, mainly TNF-α, IL-1 and IL-6; which seem to constitute the pivotal event leading to chronic inflammation. The infiltration of more immune cells increases the production of more signalling cytokines at the point of inflammation and the more is the cytokine production, more circulatory immune cells infiltrate the joint to worsen the situation. As a result, the disease reaches more severe condition as the illness-tenure progresses. As the disease course progresses, many other cytokines and chemokines are involved in RA progression, including IL-15, IL-18 and angiogenic factors. After binding to their specific receptors, these molecules regulate various signalling cascades like MAPK, nuclear factor-κB (NF-κB) or Jak/STAT signalling pathways leading to the activation or inhibition of genes responsible for mediation of inflammation and tissue degradation. Tissue-degrading enzymes like the matrix metalloproteinases (MMPs) and cell-surface molecules like selectins, integrins which are involved in inflammatory pathways, imparts striking characteristics of inflammatory responses. Following the infiltration of immune cells in the sub-lining region of the joint, the joint synovium is invaded by the immune cells and the lining layer of the cartilage-bone junction of the synovium converts into 'pannus'. Pannus is the transformation of synoviocytes into hyperplastic aggressive type of inflamed tissue containing osteoclasts. The bones start to degrade because of the accumulation of osteoclasts. The degradation of cartilage is mainly mediated by the metalloproteinase enzymes present in the joint (Redlich *et al*., 2002; Goldring, 2003).

In the arthritic synovium, the major infiltrating cells are the CD4+ T cells and macrophages which efficiently produce TNF-α. In the RA patients the TNF-α level is up-regulated and it serves as a potential factor for the production of other cytokines like IL-1, IL-6, IL-8, GM-CS etc. Blocking of TNF-α with inhibitor drugs potentially down-regulates the expression of these cytokines. TNF-α increases the proliferation of marcophages, activated T cells, B cells, synovial lining cells; induces the proliferation of different cytokines as well as collagenases and prostaglandins in the synovial lining cells (Vasanthi *et al*, 2007). TNF-α is also known to act against the suppressive activity of human regulatory T cells (T_{reg}); as a result, high TNF-α in the patient's body results in the defective T_{reg} function which normally down-regulates the cytokine production (Farrugia and Baron, 2016). Collectively, TNF-α serves as a key molecule which controls different cellular and molecular communication cascade reactions in

the synovium during inflammation and contributes significantly to the degree of disease progression. TNF-α is a prime cytokine against which different inhibitors like Infliximab, adalimumab, etanercept has been used successfully to down-regulate the arthritis (Choy and Panayi, 2001) and the molecule reserves a vital area of interest for the scientists.

Involvement of Cox in the RA progression: Increased COX-2 expression in synovial tissues is of arthritic joint is mediated chiefly by the pro-inflammatory cytokines TNF-α and IL-1. There are two isoforms of Cox: Cox-1 which is associated with general housekeeping functions and Cox-2 which is not usually detected at high level in most tissues but its expression is rapidly increased following the introduction of different inflammatory stimulus. Such stimuli include cytokines and different chemokines which stimulate COX-2 transcription by NF-κB and c/EBP activation. Additionally, signaling via cell surface integrins can also increase the expression of COX-2. Cox-2 is responsible for the production of different prostaglandins (PGs) among which Prostaglandin E2 (PGE2), is a major effector product of COX-2 in synoviocytes (**Fig. 2.8**). The PGE2 is responsible for alteration of the MMP equilibrium and increase the expression of the angiogenic factor VEGF and contribute extensively to the degree of inflammation (Crofford, 1999). Cox-2 over-expression in inflammation and RA induces pain as prostaglandins are the key effectors of pain sensation. Dilation of small blood and increased vascular permeability is induced by PGE2 which initiates inflammation; It also produces hyperalgesia by a sensitizing action on the peripheral terminals of sensory fibers.

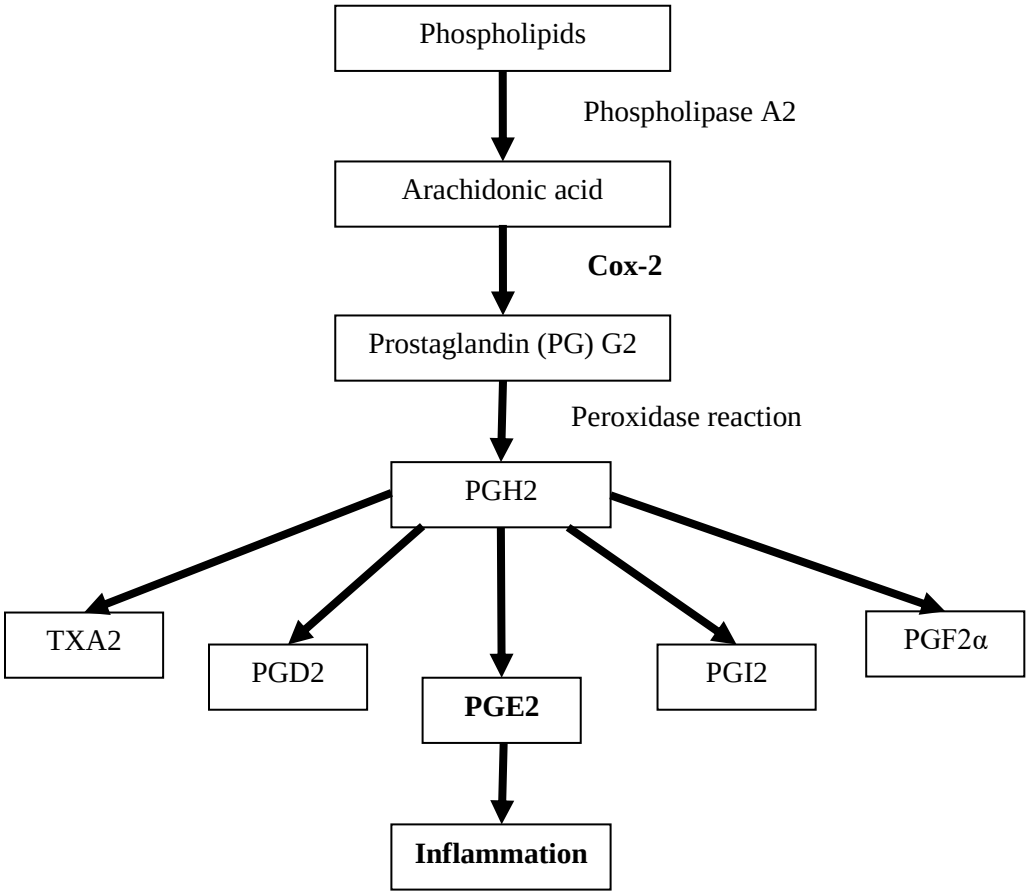


Figure 2.8. Biosynthesis of prostanoids: the role of Cox-2 in the biosynthesis of different prostanoids along with PGE2 which is a key effector molecule of inflammation.

Role of synovial fibroblasts in RA pathogenesis: Major structural characters of a synovial joint involve two components; the synovial fluid and the surrounding soft connective tissues which includes the articular cartilage, capsule and ligaments. This fluid-filled space is the site at which the articulating surfaces of the bones contact each other and attends flexibility to maneuver smoothly against each other (Smith, 2011).

Local and systemic inflammation is one of the hallmarks of RA. In the inflamed synovium, during the RA progression, the structure of the site changes and becomes a two tissue layered structure: the first layer is the outer membrane-lining intimal region and second layer is the sub-intimal region. Each of the regions shows significant changes in their architecture. In the sublining region, T-cells constitute about 30-50% of cell varieties and about 5% of sublining cells are B-lymphocytes. The proliferation of blood vessels and

lymphoid aggregation is a common character for both the intimal and subintimal regions of inflamed synovium (Bartok and Firestein 2010). Other than inflammatory cells such as neutrophils and lymphocytes, Rheumatoid arthritis synovial fibroblasts (RASF) or type-B synoviocytes contribute significantly to the various pro-inflammatory pathways within the rheumatoid joint. The macrophage-like cells display extremely activated state and produce a large array of pro-inflammatory cytokines, chemokines, and growth factors that stimulate RASFs. Specifically, RASFs in the synovium lining layer display several features of cellular activation that ultimately result in aggressive and invasive 'pannus' (Pap *et al*., 2000). The activated cell types and their secretions contribute significantly to the progression of RA. The aforementioned interactive action of synovium, immune cells and secondary lymphoid organs are briefed in the **Fig. 2.9**.

Role of reactive ions in RA progression: A growing body of evidence indicates the possible role of highly reactive products of oxygen and nitrogen, termed as free radicals, in the pathogenesis of RA as well as other degenerative diseases (Rathore *et al*., 2007). Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced endogenously during aerobic metabolism at the sites of chronic inflammation. ROS such as superoxide radical, hydroxyl radical and hypochlorous acid contribute significantly to tissue injury in RA. In addition, activated leukocytes also produce ROS. ROS can directly or indirectly damage basic articular constituents and lead to the clinical expression of the inflammatory arthritis. Synovial cavity damage correlates with fluctuating oxygen pressure in the joint, over production of ROS, lack of oxygen-processing enzymes and free radical-scavenging molecules has been reported in RA. Oxidative stress exacerbates inflammation and worsens joint tissue. The normal equilibrium between ROS production and anti-oxidant system of the cell is disturbed due to oxidative stress, thus resulting in the damage to vital cell components such as proteins, DNA and membrane lipids. There are several studies demonstrating increased levels of malondialdehyde and decrease in the activities of catalase in RA patients. Similarly, glutathione reductase activities also get disturbed in the synovial fluid of patients. Moreover, the levels of thioredoxine, which is a marker of oxidative stress, are significantly higher in the synovial fluid of RA patients. Production of nitric oxide (NO) is also up regulated in arthritic tissue (Rathore *et al*., 2007).

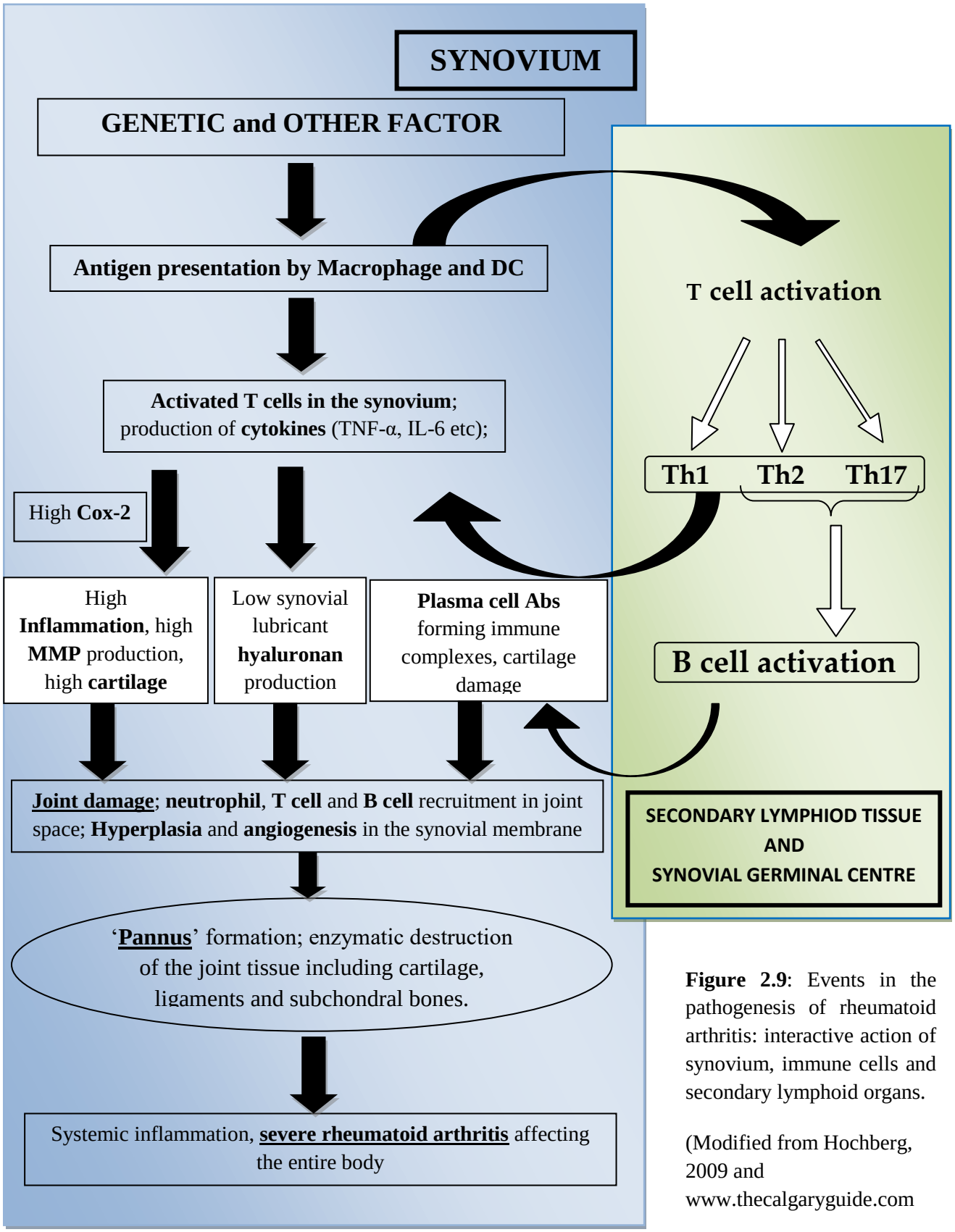


Figure 2.9: Events in the pathogenesis of rheumatoid arthritis: interactive action of synovium, immune cells and secondary lymphoid organs.

(Modified from Hochberg, 2009 and www.thecalgaryguide.com

2.2.5. Conventional medicines for RA

There are different groups of medicines used in the treatment of RA. The treatment goal is to reduce the pain and stiffness of the patient body and to improve organ function. The degree of pain is always a prime area of health in which patients want to see improvement. Non-steroidal anti-inflammatory drugs (NSAIDs) and COX-2 selective NSAIDs (known as coxibs) are, though do not belong to any distinct chemical class, but primarily works on inhibition of prostaglandin production.

Cyclooxygenases (Cox) are a group of enzymes which mediates the conversion of arachidonic acid into inflammatory prostaglandins. Prostaglandins (PGs) mediate body's response to tissue-injury or inflammation. Phospholipids are converted into arachidonic acid by phospholipase A2 enzyme; which is further converted into prostaglandin G2 by cyclooxygenase reaction; PGG2 is then converted into PGH2 through peroxidase reaction. Different isomerases of PG synthase enzymes then convert PGH2 into different prostaglandin forms including PGD2, PGE2, PGI2, PGF2α, TXA2 etc. These PGs exert autocrine or paracrine function and works through G-protein coupled receptors. Prostaglandin E2 (PGE2) is a key molecule involved in the inflammatory process and in pain signalling. PGE2 dilates the smaller blood vessels which initiates the inflammatory response and results in swelling, redness, heat and pain. It also acts on neurons and contribute to the systemic inflammatory responses like fever, pain, fatigue, hyperalgesia etc. PGs produced by Cox-1 exert the "housekeeping functions"; in contrast, Cox-2 is not detected in most tissues but its expression is rapidly increased following the introduction of inflammatory stimuli like the pro-inflammatory cytokines, lipopolysaccharides, mitogens and oncogenes, growth factors, platelet-derived growth factors, epidermal growth factors and hormones, resulting in increased PG synthesis in inflamed region (Zarghi and Afraei, 2011). NSAIDs inhibited two isoforms of Cox namely Cox-1 and Cox-2. However, the major concerns with NSAIDs are the risk of severe adverse side effects including the impairment of housekeeping function of the Cox-1 genes. The management of such side-effects were reduced after the discovery of two Cox isoforms namely Cox-1 and Cox-2 in early 1990s and the focus shifted to selective Cox-2 inhibition and retention of normal "house-keeping" functions of Cox-1 (Hawkey, 1999; FitzGerald and Patrono, 2001). The most important mechanism of action of these

medicines is focused on the inhibition of prostaglandin production by competing with arachidonic acid in the binding site of Cox-2 (Smith *et al*., 1990). The first medicine in this aspect, coxibs, a selective Cox-2 inhibitor, appeared in the market around 1999. An extensive study on NSAID, however, demonstrate that there is a slight high risk of gastrointestinal events, increased risk of thrombotic cardiovascular events (Lanas *et al*., 2007; McGettigan and Henry, 2006). Most NSAIDs compete with arachidonic acid for binding with the catalytic site of Cox-2. As a result, the formation of prostaglandin is reduced and inflammation is down regulated (Hochberg *et al*., 2009).

Glucocorticoids (GCs) are a group of steroids that works on different cytosolic GC receptors (cGCR), membrane bound GC receptors (mGCR), or acts via non-specific interactions through cell membrane (Buttgereit *et al*., 2004). GC works primarily through trans-activation or trans-repression. Furthermore, interaction of activated cGCR with different transcription factors is also recognized as an important genomic mechanism of GC action (DeBosscher *et al*., 2000; Vacca *et al*., 1992). GCs have been used efficiently against RA. GCs are lipophilic substances which can easily pass the cell membrane. To exert their effects, GCs need to bind to a specific cytoplasmic GC-receptors (cGCRs). A conserved central domain of GCR is involved in binding of GCRs directly to the to the DNA using their zinc finger domains. GCRs are about 800 amino acid residue-long, with certain areas of the molecule showing homology with other steroid receptors, thyroid hormone receptors and retinoic acid receptors. GCRs are free in cytoplasm. When glucocorticoids enter a cell by passive diffusion through the cell membrane, they bind to GCRs, changes the molecular conformation and form dimer. Upon this activation, GC-GCR complex passes the nuclear membrane and binds directly to the major groove of DNA in specific regions or interact with other transcription factors. It can also modulate the stability of specific mRNA molecule. Several steroid sensitive genes contain glucocorticoid-responsive elements (GREs) to which the GC-GCR complex binds (Van der Velden, 1998). This binding is followed either by classical cGCR-mediated genomic effects or by different other cGCR-mediated non-genomic effects. GCs can influence the transcription of approximately 1% of the total genome (Hochberg, 2009) by either ‘transactivation’ or ‘trans-repression’. The activated GC-cGCR complexes translocate into nucleus and binds with specific DNA sites known as GC-responsive elements (GREs). Trans-repression of IL-1, IL-6, TNF-α, Cox-2, phospholipase

A2 genes occur; transactivation of lipocortin-1 also takes place – all contributing to the anti-inflammatory effects. The non-genomic function includes the non-specific interaction of GCs with cell membrane. All primary or secondary immune cells are more or less affected by GCs. GCs suppress the number of circulating monocytes, macrophages, T-cells and granulocytes (Hochberg *et al*., 2009).

Disease-modifying anti-rheumatic drugs (DMARD) are another group of medicines that came into the market as an anti-rheumatic medicine for its excellent efficacy and strong safety profile (Kremer, 2004). Methotrexate, Leflunomide are two must mentioned medicines in this regard. They show a wide array of mechanism of actions and differ significantly from one another. DMARDs generally inhibit the pyrimidine synthesis pathway resulting in the down regulation of lymphocyte proliferation. Methotrexate is an analog to folic acid which is an essential co-factor for variety of enzymes essential to purine and pyrimidine synthesis. Thus, when administrated, it interferes with the ability of folic acid to serve as a co-factor for the activation of various enzymes essential for the production of purine and pyrimidine. As a result, it inhibits the proliferation of different immune cells in inflamed condition (Breedveld and Dayer, 2000). Another DMARD namely leflunomide downregulates *de novo* pyrimidine synthesis through inhibiting dihydroorotate dehydrogenase, a rate limiting factor resulting in a pyrimidine crisis in lymphocytes (Breedveld and Dayer, 2000). Activated lymphocytes expand their pyrimidine pool approximately 8 folds during proliferation. In contrast, purine pools are increased approximately 2 folds. Thus, inhibition of dihydroorotate dehydrogenase prevents lymphocyte from accumulating sufficient pyrimidines to support DNA synthesis and exert immunomodulatory function. However, there are other DMARDs like hydroxyl-chloroquine, chloroquine, sulfasalazine, tetracycline-derivatives, gold, azathioprine, cyclosporine and many more. There is good evidence that other DMARDs alone or in a combinational way, help the improvement of RA and its management (O'dell, 2001).

Different cytokine inhibitors have been approved as a remedy in RA in recent times. Drugs to control the cytokine network seem to be advantageous in the inhibition of disease progression. Different experiments and clinical trials on animal models and humans have provided the scientific community useful information about the role of cytokines and other biomolecules in RA (for example, Cox-2) as well as the effect of their inhibition by different

drugs. Some are being developed and undergoing different clinical trial phases. Infliximab, adalimumab, etanercept are some of the TNF-α inhibitors being used in the medication. For infliximabs, the exact mechanism of action is not well understood expect the direct activity of the molecule that neutralizes cytokines. The compound is purified; recombinant-DNA derived chimeric human-mouse IgG monoclonal antibody that consists of mouse heavy and light chain variable regions combined with human heavy and light chain constant regions (Akiho *et al*, 2015). It neutralizes the biological activity of TNF-α by binding with the high affinity to the soluble or membrane-bound forms of TNF-α, and inhibits the effective binding of the TNF-α with its receptors (Choy & Chung, 2001). IL-1 receptor antagonists like anakinra is also in use. Another group of medications include tocilizumab, which is humanized anti-human IL-6 monoclonal antibody which specifically targets IL-6; rituximab, a high-affinity chimeric monoclonal antibody specific to CD20 which helps in the depletion of B cells interacting in the inflamed joint (Hochberg *et al*., 2009).

2.2.6. Animal models of RA

Several animal models have been established to conduct experiments regarding the pathogenesis and treatment of auto-immune arthritis. These models have striking similarities with the clinical, immunologic and histologic characteristics of the human RA, although no animal model fully replicates all the features of RA. But these models significantly contribute to the research fields of RA. The most commonly used model animals are the rodents. There are two distinct categories of animal models: one is experimentally induced and the other one is spontaneously induced disease model. Arthritis can be induced in susceptible mouse or rat strains through introducing microbial products, joint-specific antigens, routine test antigens and many other compounds. Alternatively certain mouse strains that develop spontaneous arthritis serve as the experimental model as well. The brief account of different arthritic models has been given in the **Table 2.4**.

Table 2.4: A brief account of some efficient rodent arthritic models used in RA-associated research (modified from Hochberg, 2009).

Experimentally-induced arthritis				
Model	**Preferred Rat/ mouse Strains**	**Sex bias**	**RF presence**	**References**
Adjuvant-induced arthritis (AIA)	Lewis, Wistar albino rats	-	-	Taurog *et al*., 1988; Newbould 1963.
Streptococcal cell wall-induced arthritis SCWIA)	Lewis rats	+	-	Cromartie *et al*., 1977
Collagen-induced arthritis (CIA)	Lewis rats	-	-	Ridge *et al*., 1988
Spontaneously-induced arthritis				
Rat/ mouse strain	***Arthritogenic process***	***Sex bias***	***RF presence***	***References***
K/BxN	Abs against glucose-6-phosphate isomerase	-	-	Korganow *et al*., 1999
SKG	Mutation in SH2 domain of ZAP 70 resulting in thymic selection defect	+	+	Sukaguchi *et al*., 2003
BALB/cA IL-1Ra$^{-/-}$	Deficiency in IL-1Ra	-	+	Zhou *et al*., 2005

2.2.6.1. Adjuvant-induced arthritis in rodents

Adjuvant-induced arthritis (AIA) is a polyarthritis unique to different rat models. The model has a wide acceptance in the research domains of RA and was accepted as the model for the anti-arthritic studies in this thesis (Newbould, 1963; Taurog *et al*., 1988). AIA is generally achieved by injecting Freund's complete adjuvant (FCA or CFA) in the inter-plantar region of the hind paw of experimental rats. FCA contains high concentrations of heat-killed *Mycobacterium tuberculosis* in mineral oil. The paw joint swelling develops within 4-5 days and disease develops prominently within 10-12 days. Booster doses can be introduced to flare up the inflammation in later phases of disease progression. This disease model is characterized by swellings in the inflamed joint, erythema, tenderness in the joints

of hind-paw and forepaws. There is no significant influence of sex of the animal on disease progression. Histological sections of the affected joints demonstrate synovial inflammation, pannus formation with mononuclear cell infiltration, cartilage and bone erosions, ankylosis and even granuloma formation in periarticular tissue. AIA is a T cell mediated autoimmune disease. The features of this model are reliable, robust and easily measurable. Cartilage destruction occurs but remains mild in comparison to the degree of inflammation and bone destruction of the inflamed joint (Bendele 2001).

Animal testing of new products of formulations in rodents and other subhuman primates are essential pre-requisites for conducting detailed pre-clinical and clinical trials on RA patients. Any protocol for the conductance of such processes have been validated and standardized by different authorities including Food and Drug administration (FDA). However, strict monitoring of animal ethical committees and other ethical formalities are to be considered, validated and maintained by the experimenting authorities. Especially experimentally-induced arthritic models have been the mainstay of preliminary screening of anti-arthritic agents and related toxicity tests (Hochberg, 2009).

2.3. *Aloe Vera*: the subject plant

Aloe vera is one of the most commonly used plants in the field of herbal medicine. The plant has been used by different ethnic populations all around the world. The plant is naturalized in the Indian subcontinent with wide distribution. The use of this plant has been well recorded in the ancient Indian, Greek, Roman, Chinese and Egyptian culture and its medicinal properties have covered a wide variety of disease symptoms. The name of the genus *Aloe* comes from the ancient Arabic word "Alloeh" which means bitter and shining substance, referring to its gel like parenchymal part of the inner leaf. The scientific name of *Aloe vera* (L.) Burm f. is validated by the International Rules of Botanical Nomemcleture as the legitimate name for the species along with its synonyms like *Aloe barbadensis* Mill, *Aloe chinesis* Bak etc. (Tucker 1989; Ahlawat and Khatkar,2011). The systematic position of the plant is as follows:

Kingdom	Plantae
Subkingdom	Tracheobionta
Superdivision	Spermatophyta
Division	Magnoliophyta
Class	Liliopsida
Subclass	Liliidae
Order	Liliales
Family	Aloaceae
Genus	Aloe L.
Species	*Aloe vera* (L.) Burm. f.

Synonym: *Aloe barbadensis* Miller; *Aloe vulgaris* Lam; *Aloe chinesis* Bak

Classification of *Aloe vera* (L.) Burm. f. following the PLANTS database. (https:// plants.usda.gov 20 June 2020), National Plant Data Team, Greensboro, NC 27401-4901. USA)

2.3.1. Description of the plant

The plant is a stem-less or short-stemmed plant, succulent in nature (**Fig. 2.10**). The plant has fleshy thick leaves, generally green to grey-green, grows up to 60-100 cm and spreads by offsets. *Aloe vera* is a perennial clump forming plant, having large basal leaves, usually 12-16 per plant and with a thick fibrous root. The plant has a weight of about 1.5 kg

when mature and shows a life span of approximately 12 years. The margin of the leaves contain very small teeth-like serrated edge. The flowers are generally produced in summer, generally spikes up. Some varieties of the plant shows white flecks on their leaf and lower stem surfaces. The thick fleshy leaves are made up of gel-like parenchyma layer (Grindlay and Reynolds 1986; Eshun and He 2004; Surjushe *et al*., 2008).

Figure 2.10. The *Aloe vera* plant.

2.3.2. Distribution of the plant

The plant is known to be originated in the tropical regions of Africa and it is now naturalized in warm climatic areas of Asia, Europe and America. The species is cultivated for its economic values as well (Harding, 1979). The plant is well distributed globally in all tropical and sub-tropical regions including Asia, Africa, South America, Southern part of North America and Australia. In India, the plant is naturally grown in North-Eastern India and sub-Himalayan region. The plant is seen in the southern part of the country as well. The

medicinal plant garden of the University of North Bengal has maintained this plant. The plant is naturally grown in the different areas of the campus and in the adjacent areas as well (**Fig. 2.11**).

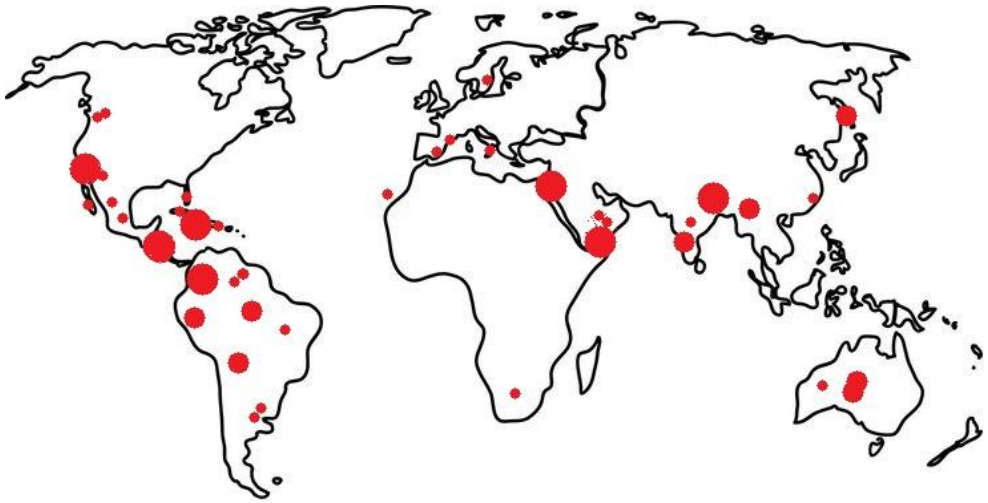


Figure 2.11. Global distribution of the plant *Aloe vera*. (Modified from Chinchilla *et al*., 2013; www.discoverlife.org)

2.3.3. Major constituents of the plant

Aloe vera whole leaf, including the gel and latex, contains more than 200 phytocompounds (Davis, 1997). The gel contains more than 98% of water (Bozzi *et al*., 2007). The solid content of the gel is about 0.66% and about 0.56% solid content is soluble in different solvents. However, the chemical composition, potency and amount of the constituents are influenced by changes in the seasons, soils, climatic conditions, extraction and harvesting methods and shows fluctuation (Boudreau and Beland, 2006; Rodriguez Rodriguez *et al*., 2010). The growth stages also contribute to its phytochemical constituents (Hu *et al*., 2003). The solid portion of the gel consists of about polysaccharides (~55%), sugars (~17%), minerals (~16%), proteins (~7%), lipids (~4%) and phenolic compounds (~1%) (Luta and McAnalley, 2005) (**Fig. 2.12**).

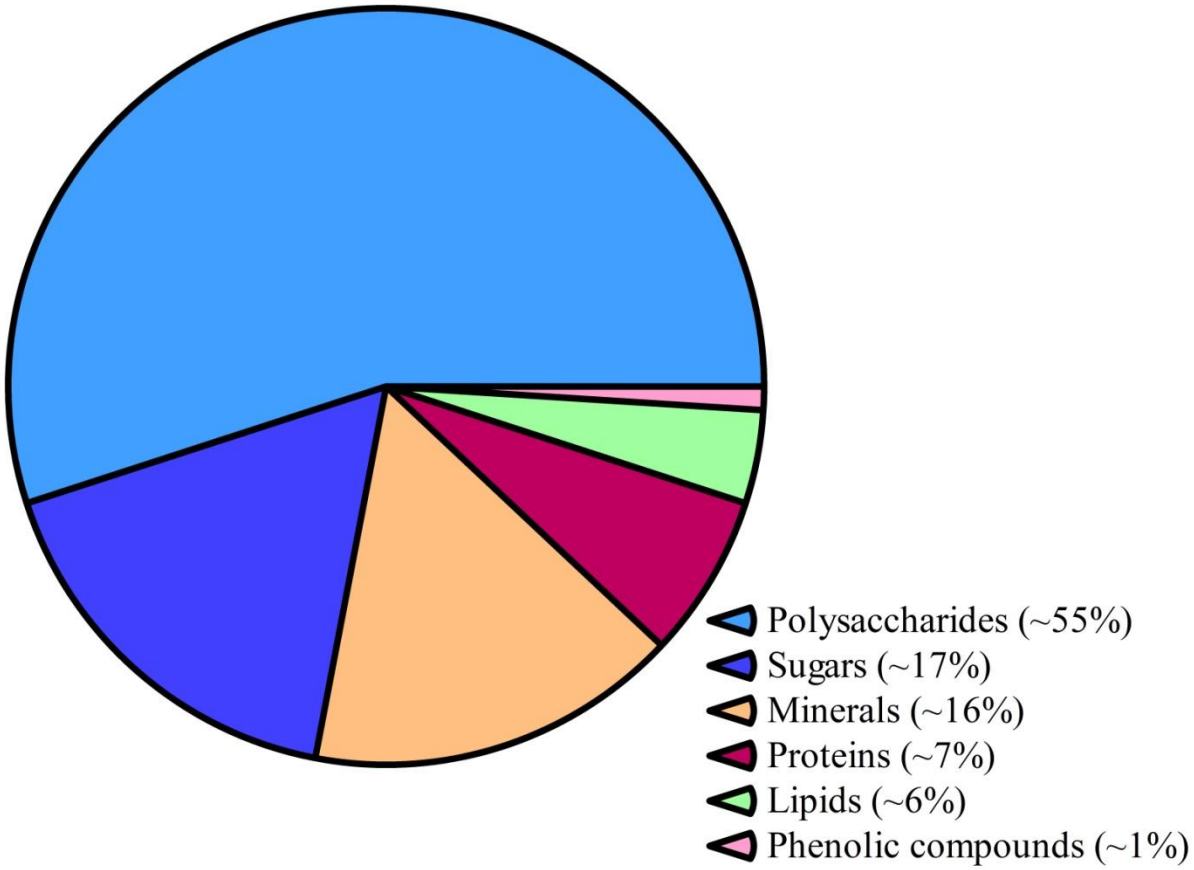


Figure 2.12. Chemical composition of *Aloe vera* gel (on dry weight basis) (Modified from Luta and McAnalley 2005).

It contains vitamin A, C, E, thiamine, niacin, riboflavin, choline and folic acid as well (Lawless and Allen, 2000). Vitamin B12 is also found in trace amounts which are generally available from animal sources (Coats 1979; Atherton, 1998). A detailed account of the compounds has been provided in **Table 2.5**.

Table 2.5: Summary of the phytochemicals of *Aloe vera* pulp and exudates (Ni and Tizard, 2004; Dagne *et al*., 2000; Femenia *et al*., 1999; Choi and Chung, 2003).

Group	Compounds
Anthraquinones/anthrones	Aloe-emodin, aloetic-acid, anthranol, aloin A and B (or collectively known as barbaloin), isobarbaloin, emodin, ester of cinnamic acid
Carbohydrates	Pure mannan, acetylated mannan, acetylated glucomannan, glucogalactomannan, galactan, galactogalacturan, arabinogalactan, galactoglucoarabinomannan, pectic substance, xylan, cellulose
Chromones	8-C-glucosyl-(2′-O-cinnamoyl)-7-O-methylaloediol A, 8-C-glucosyl-(S)-aloesol, 8-C-glucosyl-7-O-methyl-(S)-aloesol, 8-C-glucosyl-7-Omethylaloediol, 8-C-glucosyl-noreugenin, isoaloeresin D, isorabaichromone, neoaloesin A,
Enzymes	Alkaline phosphatase, amylase, carboxypeptidase, catalase, cyclooxidase, cyclooxygenase, lipase, oxidase, phosphoenol, pyruvate carboxylase, superoxide dismutase
Minerals	Calcium, chlorine, chromium, copper, iron, magnesium, manganese, potassium, phosphorous, sodium, zinc
Lipids and miscellaneous organic compounds	Arachidonic acid, γ-linolenic acid, steroids (campestrol, cholesterol, β-sitosterol), triglicerides, triterpenoid, gibberillin, lignins, potassium sorbate, salicylic acid, uric acid
Amino acids	Alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, hydroxyproline, isoleucine, leucine, lysine, methionine, phenylalanine,proline, threonine, tyrosine, valine
Proteins	Lectins, lectin-like substance
Saccharides	Mannose, glucose, L-rhamnose, aldopentose
Vitamins	A, C, E, B1, B2, B6, C, β-carotene, choline, folic acid, α-tocopherol

2.3.4. Ethnic and medicinal usage of the plant

From Mesopotamian period dated 1750 B.C.E., *Aloe vera* has been documented as a pharmaceutical remedy. In the ancient Egyptian books (550 B.C.E.), *Aloe vera* has been mentioned as a remedy against skin infections. In 74 B.C.E., Greek physician Dioscorides, in his book *De Materia Medica*, described Aloe as a wound healer, infection healer, hair-loss preventive measure and hemorrhoid eliminator (Davis, 1997). The documentation of oral and topical use of *Aloe vera* has been created since the ancient period and has been explored for its different properties in different time. With the emergence of evidence-based complementary and alternative medication, *Aloe vera* had become one of the key interests of the researchers for its wide array of efficacy. The first credible report regarding the therapeutic use of *Aloe vera* was documented in 1935 as a remedy against radiodermatitis, when the gel was applied topically (Collins and Collins, 1935). Subsequent similar cases were documented in the following years (Wright, 1936).

The second-degree thermal burn was also successfully ameliorated using *Aloe vera* gel (Tchou, 1943). Some of the first reports on the anti-inflammatory property of genus *Aloe* in rat model were reported in 1982, where the *Aloe vera* has been used as a pharmaceutical remedy in rat burn wounds, anti-prostanoid activity of the gel was thought to be a important for imparting its anti-inflammatory activity (Robson, 1982). Hanley reported in 1982 that *Aloe vera* gel could down-regulate the inflammatory response in adjuvant-induced rats (Hanley, 1982)

In the Indian medicinal systems, the plant enjoys a great degree of popularity. The Sanskrit name of *Aloe vera* ‘Ghrita kumari’ contains the term ‘kumari’ which means young girl as it is believed to bring back youthful energy and femininity (Lanka, 2018). Aloe is also used as a tonic for the female reproductive system. According to Ayurvedic documentations, *Aloe* has been used as an appetite-stimulant, purgative, diuretic, laxative and against cough, cold, piles and different other diseases. People from Tamil Nadu and from many other regional cultures of India use Aloe as a food (Ghazanfer, 1994; Heber, 2007).

Worldwide, different other prospects of this medicinal plant have been explored in the recent decades which have made the plant more useful for medicinal uses. *Aloe vera* gel

has been seen to reduce alcohol-induced acute gastritis in experimental animals (Park *et al*., 2017). It has also been used experimentally to increase the salivary flow rate in male Wistar albino irradiated model (Nejaim *et al*., 2014). When treated with *Aloe vera*, the incision wound of Wistar rats were healed and increased production of fibroblasts and TGF-β was observed (Takzaree *et al*., 2016). Improvement in the organization of skin and collagen was also observed in a different study on the same model (Brandão *et al*., 2016). In the alloxan-induced diabetes with wound models, *Aloe vera* gel was used to down-regulate the production of necrotic tissue and inflammation. Wound areas were healed significantly and better re-epithelialization was observed (Sari *et al*., 2018). In the X-ray irradiated male Balb/c mice, the hepatic and renal function parameters were improved, ROS production was reduced, lactate dehydrogenase and lipid peroxidation activity was down-regulated after treating with *Aloe vera* aqueous extract (Bala *et al*., 2018). In the acetaminophen-induced hepatitis models, *Aloe vera* was seen to reduce the expression of IL-12 and IL-18 cytokine, ALT transaminase and also reduced hepatitis (Werawatganon *et al*., 2014). The *Aloe vera* extract is also seen to reduce the inflammatory Cox-2 and VEGF levels in breast cancer mice model (Shirali *et al*., 2017). Different disease parameters including blood glucose, insulin level, lipid alteration, oxidative stress, neuronal loss in hippocampus were brought back to normalcy in the streptozotocin-induced rat models after treating with *Aloe vera* extract (Noor *et al*., 2017; Arora *et al*., 2019) and crude gel (Tabatabaei *et al*., 2017). *Aloe vera* has been a curative remedy in different ischemia-reperfusion injury models established in rats and has provided significant cardio-protective effect as well (Guven *et al*., 2016; Sahin *et al* 2017). Remarkable activity of Aloe-emodin, an *Aloe vera* gel compound has been observed in cell culture of MH7A human RA synovial fibroblast-like cells where the compound has provided protection against rheumatoid arthritis by down-regulating viable cell numbers and inducing apoptosis (Hashiguchi *et al*., 2017). It is said to be a complementary treatment to the conventional medicines like methotrexate.

Apart from different extracted or purified phyto-compounds of *Aloe vera* (**Table 2.5**), the naturally harvested crude unprocessed *Aloe vera* gel has not been explored for its medicinal properties. Works on crude unprocessed gel, however, has shown that the crude gel is efficient in animal models (Tabatabaei *et al*., 2017) which show its effectiveness on a wide spectrum of behavioural deficits, anxiety-like behaviours, cognitive and exploratory

properties, depression-like behaviour, locomotory tiredness, weight loss and in the experimental diabetic rats.

2.5. Cytokine targeting drug and herbal medicine: New era of RA prevention

As discussed earlier, the cytokines and Cox-2 constitute the most important communication pathway in the pathogenesis of RA which has an in-depth role in the progression of arthritic condition. TNF-α, different interleukins and other chemokines are responsible for the infiltration of immune cells at the inflamed joint (Feldmann *et al*., 1996). In a detailed study on rat knee, the TNF-α and IL-1β was seen to launch inflammation. A dose of 10 µg of TNF-α injected in the knee joint space of hind paw produced significant increase in the inflammatory symptoms and cartilage matrix destruction (Bolon *et al*., 2004). Cox-2 plays pivotal role in the inflammation by promoting prostaglandins which evoke inflammatory pain (Goldenberg 1999). It has been seen in clinical trials that different cytokine inhibitors and (Maini and Taylor, 2000) Cox-2 inhibitors (Goldenberg 1999) has efficiently down-regulated the progression of RA when used alone or along with other conventional drugs. The usefulness of these findings is associated with the emerging concept of targeting cytokine networks using drugs to ameliorate RA (Maini and Taylor, 2000).

Beside the conventional therapies and drugs, different plant extracts and plant-based products have showed effective interaction with cytokines *in silico*. Known phyto-compounds from *Cannabis sativa*, *Prunella vulgaris* and *Withania somnifera* has been has been seen efficiently binding with different cytokines *in silico* (Zaka *et al*., 2017). Different isolated biomolecules from genus *Phyllanthus* (Family Euphorbiaceae) has bound efficiently with Cox-2, PGE synthase, TNF-α and IL-1β, and the NMDA receptor (Chopade *et al*., 2015). Compounds present in *Aloe vera* (kaempferol), *Arctium lappa* (arctiin), *Camellia sinensis* (epigallocatechin gallate), *Capsicum annuum* (capsaicin), *Chamaemelum nobile* (apigenin), *Curcuma longa* (curcumin diglucoside, curcumin monoglucoside), *Matricaria chamomilla* (chamazulene), *Matricaria recutita* (bisabolol), *Myrica cerifera* (myrcetin), *Senegalia catechu* (catechin), *Sophora japonica* (sophoricoside), *Syzygium aromaticum* (eugenin), *Tagetes lucida* (isorhamnetin) and *Wikstroemia indica* (daphnoretin) also have been used in molecular docking and has shown efficient binding affinity with cytokines like

TNF- and IL-1 (Xu *et al*., 2018). *Aloe vera* based phytocompounds like kaempferol, Aloesin, barbaloin, aloe-emodin and other anthroquinones have proved efficient in interaction with TNF- α, NF-κB , MMP-9, COX-2 *in silico* (Kshirsagar *et al*., 2014; Xu *et al*., 2018; Tripathi *et al*., 2018).

Along with the *in silico* findings, different *in vivo* approaches using appropriate animal models or cell culture-base studies are also successful to establish the fact that plant based compounds can effectively interact with the cytokine network thereby affecting the progression of RA. The different herbal crude products, extracted in aqueous medium or in different solvents have shown promising role in amelioration of inflammation and inflammatory diseases. Aqueous extract of *Trachyspermum ammi* seeds were seen to actively down-regulate the Cox-2 and iNOS expression in the cartilage of the collagen-induced arthritic rats along with other physiological parameters (Korani and Jamshidi, 2020).Treatment with *Withania somnifera* root extract was shown to down-regulate iNOS, NF-κB, TNF-α, MMP-8 mRNA expression in the synovial tissue of arthritic rats (Khan *et al* 2019). Anti-arthritic properties of crude extract of *Piptadeniastrum africanum* have been evaluated in FCA-induced arthritic rat models and it was shown to down-regulate TNF-α and IL-2 expression; the plant extract also decreased the proliferation of T cells and ROS production when administrated *in vivo* (Mbiantcha *et al*., 2017). *Moringa rivae* leaf extract is seen to down-regulate the transcription of TNF-α, Cox-2 and different other cytokines in the FCA-induced inflammatory arthritic rats (Saleem *et al*., 2020). Fekugreek (Trigonella foenum graecum) seeds extracted in ethanol significantly brought back different arthritic parameters towards normalcy including ESR, TC WBC, DC WBC, RBC, haemoglobin counts and down-regulated the production of cytokines like TNF-α in the arthritic animal models (Suresh *et al*., 2012). Dried gum resin of Boswellia carterii has shown potential down-regulation of TNF-α, IL-1β and also decreased arthritic paw edema and other arthritic parameters in adjuvant-induced inflammatory rats (Fan *et al*., 2005). Xanthium strumarium fruit, a widely used medicinal remedy in China was also effective against adjuvant-induced arthritic rats regarding the down-regulation of TNF-α and Cox-2 and for lowering the arthritic paw swelling (Lin *et al*., 2014). The plant parts of *Ephedra gerardiana* extracted in different solvents were observed to attenuate FCA-induced arthritis in Sprague Dawley rats by down-regulating different cytokines and Cox-2. Poly-herbal formulations like Kashayams

were used to treat the arthritic rat models. It controlled the elevated levels of Cox-2, TNF-α, iNOS mRNAs *in vivo* (Aswathy *et al*., 2021). In the cell-culture based experimentation, LPS-induced mice macrophage RAW 264.7 cell line, when incubated with phytocompounds obtained from *Litsea cubeba*, showed down-regulation of iNOS and Cox-2 which were elevated in the cultured cells due to LPS induction (Lin *et al*., 2016). Agnuside, a compound isolated and purified from the leaf extract of a plant *Vitex negundo* was orally administrated in adjuvant-induced polyarthritic Wistar rats and a large array of pro-inflammatory mediators and cytokines were assessed through flow cytometry. Those molecules are known to have increased expression in inflammatory arthritis. It was seen that the pro-inflammatory cytokines (IL-2, TNF-α, IL-4, IL-10, IL-17) and pro-inflammatory mediators (Prostaglandin E2 and leukotrine B4) were down regulated in the agnuside treated animals in a dose dependent manner (Pandey *et al*. 2012).

A detailed exploration of the plant *Aloe vera* and its crude gel, however, has not been done in detail in appropriate animal models and it is required to draw a confirmatory conclusion about the role of this plant and its constituents against the inflammatory symptoms of RA.

The efficacy of botanical products and plant extracts in the prevention of inflammation and RA produce the foundation knowledge about the importance of bio-prospecting for newer synergistic combinations and pharmacological agents. In different cases, ‘Traditional’ medicinal systems recommend complex mixtures of herbs or crude part of the plant which holds an approach to control different multi-factorial causes such as chronic and degenerative ailments. Compared to ‘one disease- one target- one drug’ concept of biomedicine, traditional medicinal systems bank on the concept that, a mixture of moderately active metabolites present in an extract would be potentially able to interfere and regulate different proteins on the same signaling pathway leading to synergistic pharmacological effects (Leonti and Casu, 2013). There have been studies on several herbal remedies about synergism and antagonism, pharmacodynamics, pharmacokinetics, dosing and interactions; however, more explorations are required in depth. Traditional knowledge, particularly from the great traditions of Ayurveda and Traditional Chinese medicine will have an important role in bio-prospecting. A golden triangle strategy, which integrates

modern medicine, traditional knowledge and the robust use of science and technologies with a systems biology approach, is an efficient approach to open up new opportunity windows for herbal medicine and CAM therapy.

The Golden triangle approach (Patwardhan and Gautam, 2005) for finding new alternative remedy against inflammatory arthritis and other inflammatory conditions using herbal and natural resources has been the background of this present thesis (**Fig. 2.13**).

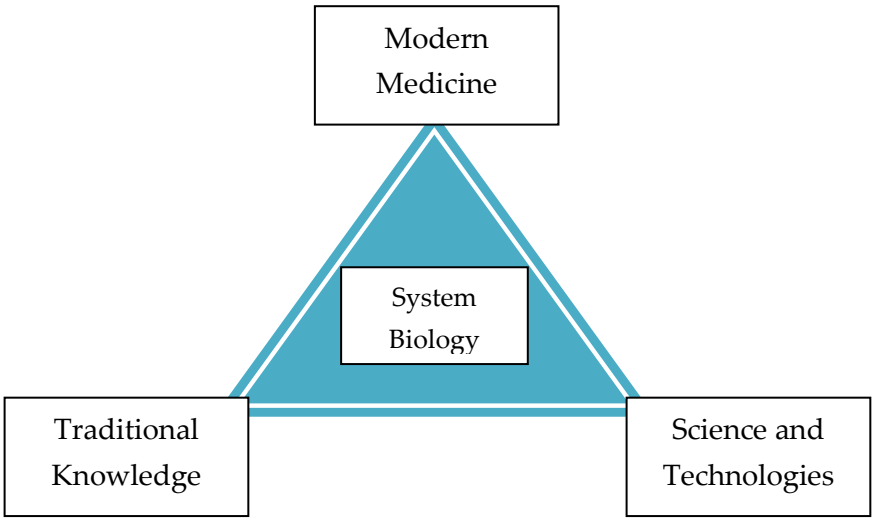


Figure 2.13. Golden triangle approach. It integrates traditional wisdom, contemporary science and technology, and the evidence based of modern medicine, where the holistic strategies are reflected from the principles of systems biology (Patwardhan and Gautam, 2005).

Using the traditional knowledge on *Aloe vera*, I have tried to explore the role of this plant against inflammation and RA in suitable animal models. Crude plant and poly-herbal formulations are known to have better efficacy in Ayurveda due to the synergistic role of different components present in the different plants (Leonti and Casu, 2013, Parasuraman *et al*., 2014). The traditional medicinal systems also encourage the use of multiple metabolites present in a single plant extract or in a mixture of plants to interfere with different physiological pathways resulting in a complete cure (Leonti and Casu, 2013). The same principle has been kept in consideration during the exploration of the subject plant in the inflammatory arthritic condition. The crude unprocessed homogenized extracts have been

used in the present dissertation to investigate the efficacious role of the *Aloe vera* gel against inflammation and inflammatory arthritic condition.

OBJECTIVES OF THE STUDY

The objectives of the thesis remain as follows:

1. To standardize the crude extract, aqueous and alcoholic extraction procedure of *Aloe vera* crude gel along with in vivo toxicity tests and dose determination.

2. To investigate the anti-inflammatory potential of extracts of *Aloe vera* crude gel in anti-inflammatory models in Rats.

3. To evaluate the anti-arthritic properties of extracts of Aloe vera crude gel in rheumatoid arthritic model in rats.

4. To analyze the expression profiles of few related cytokine genes in the rheumatoid arthritic rat models in response to *Aloe vera* crude gel by Real time Reverse Transcriptase PCR method.