1. INTRODUCTION

Rheumatoid Arthritis (RA) is a chronic, progressive inflammatory disorder characterized by inflammation and cellular proliferation in the synovial lining of joints that can ultimately result in cartilage and bone destruction. RA is also the most common form of inflammatory arthritis in adults, affecting approximately 0.3 - 1.2% of world’s populations (Carmona et al., 2002); despite intensive investigations the etiology of RA remains undefined.

Diagnosis is based on symptoms related to synovial inflammation, the presence of rheumatoid factor (RF) and radiological signs of joint damage (Arora et al., 2000b). It is believed that RA results from the exposure of the genetically susceptible host to relevant, but currently unidentified, etiopathogenic antigen (Sewell and Trentham, 1993). Dysregulation of various components of the immune response has been demonstrated in affecting patients and is thought to contribute to this systematic inflammation.

The symptoms of rheumatoid arthritis come and go, depending on the degree of tissue inflammation. When body tissues are inflamed, the disease is active. When tissue inflammation subsides, the disease is inactive (in remission). Remissions can occur spontaneously or with treatment and can last weeks, months, or years. During remissions, symptoms of the disease disappear, and people generally feel well. When the disease becomes active again (relapse), symptoms return. The return of disease activity and symptoms is called a flare (Imboden et al., 2007). The course of rheumatoid arthritis varies among affected individuals, and periods of flares and remissions are typical. When the disease is active, symptoms can include fatigue, loss of energy, lack of energy, low-grade fever, muscle and joint aches, and stiffness. Muscle and joint stiffness are usually most notable in the morning and after periods of inactivity. Arthritis is common during disease flares. Also during flares, joints frequently become red, swollen, painful and tender. This occurs because the lining tissue of the joint (synovium) becomes inflamed, resulting in the production of excessive joint fluid (synovial fluid). The synovium also thickens with inflammation (synovitis) (Coico and Sunshine, 2009).

Complement is one of the most powerful effector system involved in the body's defense. It is a pro-inflammatory system consisting of about 30 proteins in the form of inactive enzyme precursors, soluble or cell-bound regulatory proteins and receptors specific for effector peptides of the system. Effector peptides like C5a, a very powerful anaphylatoxin and immune complexes work additively towards the induction of
Introduction

immune-complex induced arthritis. The phenomenon is referred to as “Complement activation” (Carroll, 2004). The activation is brought about by (a) antigen-antibody complex through the classical pathway, (b) by antigen only through an alternative pathway and (c) MBL (Mannose Binding Lectin) triggered lectin pathway. Proinflammatory anaphylatoxins (C3a and C5a), chemotaxins (C5a), opsonins (C3b, C4b, iC3b, C3d, C3dg) and a terminal lytic complex C5-C9, known as Terminal Complement Complex (TCC) or Membrane Attack Complex (MAC) are generated. These pro-inflammatory peptides are vital for host immunity against infection but may be highly injurious to self if the activation goes uncontrolled (Gerald, 2006).

RA involves all elements of the immune response. It is initiated by immune complexes (ICs) and complement, perpetuated by cytokines and affected by metalloproteinases (Imboden et al., 2007). The local production of IgG and rheumatoid factors (RF), which are auto antibodies directed against the Fc portion of IgG, along with the antibodies to anti-cyclic citrullinated peptide lead to complement activation, appear important in destructive events associated with the synovitis (Shibuya et al., 1991). There is strong evidence that both the classical and the alternative pathways of the complement system are pathologically activated during RA (Reid and Turner, 1994). Complement activation contributes to a pathological process in a number of autoimmune and inflammatory diseases, including RA (Rynes et al., 1982; Frank et al., 1987). The classical pathway can be initiated by several triggers present in the inflamed joint such as deposited autoantibodies, dying cells, and exposed cartilage proteins such as fibromodulin. B cells producing autoantibodies, which in turn form immune complexes, contribute to RA pathogenesis partly via activation of complement (Larsen et al., 2006). Complement system thus may be highly injurious to self if the activation goes uncontrolled. Activation of complement system is tightly regulated by complement proteins (CRP). Some of these proteins called RCA (Regulators of Complement Activation) are encoded by a single gene cluster. Out of these proteins complement receptor 1 (CR1) and complement receptor 2 (CR2) have multiple roles that define them as key complement proteins in the onset of autoimmune disorders like RA. The experimental evidence and functional attributes of CR1 and CR2 suggest their immense role in the immune-imbalance in autoimmune disorders and significance as putative disease markers.

Complement Receptor 1 (CR1), the receptor for complement peptides C3b and C4b is a key component of the complement system. CR1 is a single chain glycoprotein having an extracellular domain of 1930 residues, a transmembrane domain of 25 residues and a 43 residue cytoplasmic domain (Klickstein et al., 1987). CR1 is a polymorphic protein,
molecular weight ranging from 190 to 280 KD. CR1 is found on the surfaces of erythrocytes (E-CR1), most peripheral blood leucocytes (L-CR1), glomerular podocytes (G-CR1) and, follicular dendritic cells. CR1 is also present in soluble form in plasma (the soluble CR1, sCR1) and in urine (the urinary CR1, uCR1). Of all the complement receptors, CR1 gained much more attention due to its multiple functions in the immune system and, modulation of its levels in autoimmune and inflammatory disorders. CR1 is the most effective complement regulatory protein (CRP). It regulates complement activation by removing circulating immune complexes and inhibiting the cascade activation. E-CR1 is the main vehicle for IC clearance (Sivasankar et al., 2004). Recent investigation suggests a regulatory role of CR1 present on lymphocytes. CR1 prevents and interrupts the cascade activation by binding with C3b and C3d, facilitating their decay and also by acting as a cofactor for factor ‘I’, a serine protease that degrades complement peptides to smaller fragments thereby controlling or destroying their activities. CR1 is the only CRP that has the cofactor and decay accelerating activity for the inactivation of the classical as well as the alternative pathway of complement activation. CR1 facilitates uptake and clearance of Antigen-Antibody complexes, antigens and apoptotic bodies by phagocytes. Role of CR1 in the regulation of B and T-cell activation, immune tolerance, host-pathogen interactions and survival of intracellular pathogens are also suggested (Fingeroth et al., 1989). Over the years CR1 has been in the focus of research aimed at elucidating the pathophysiology of inflammatory and autoimmune disorders. This has been more than justified since any qualitative or quantitative impairment of CR1 could disrupt the immune homeostasis especially at the effector end of immune responses (Fingeroth et al., 1989). Autoimmune disorders like SLE and RA are present with IC overload and excessive generation of pro-inflammatory complement peptides. This suggests inadequacy of complement regulation and immune complex clearance in these diseases. Several studies have shown modulation of CR1 levels in the above diseases. CR1 was found to be up regulated on neutrophils in synovial fluid samples from patients with inflammatory joint disease with rheumatoid arthritis (RA) and with other articular diseases (OAD). A reduction in the expression level of CR1 on erythrocytes has been observed in SLE, RA and kidney disorders. A decline in B-cell and neutrophil CR1 had also been reported (Jozsi et al., 2002). It has been suggested that decline in E-CR1 seen in SLE may aggravate immune complex (IC) overload due to non-clearance and exaggerated complement activation due to impairment of complement regulation (Hebert, 1991). Studies also suggested inverse relationship between the levels of E-CR1, L-CR1 transcript and SLE disease activity.
Levels of L-CR1 transcript got up regulated in those patients who entered remission and declined further in the patients with poor prognosis. Inheritance, enhanced proteolysis and reduced CR1 gene transcription are mechanisms suggested to explain the decline in the levels of CR1 on the cell surface. These patients had the worst prognosis; this immensely suggested the usefulness of CR1 as a disease marker (Arora et al., 2004). These findings suggest that up regulation of CR1 may be an effective therapeutic strategy for such diseases. Each of these aspects needs to be further verified. In this direction, the prime requisite is to understand the regulation of CR1 expression under normal physiological conditions. Due to major focus on modulation of E-CR1 in disease and enhanced proteolysis as the widely accepted reason for decline of E-CR1, regulation of CR1 expression in nucleated cells was not well investigated. Studies on L-CR1 had geared up only recently. The levels of CR1 transcript correlated with the levels of CR1 proteins.

To summarize, CR1 is a complement receptor that prevents injurious effects of the complement and immune complexes in the host and maintains immune homeostasis by regulating the adaptive and innate immunity and, effector functions of the immune system. The level of CR1 either increases or decreases in autoimmune and inflammatory disorders. This makes CR1 as a putative disease activity marker. Very little is known about the factors that modulate CR1 levels in autoimmune diseases or those which maintain CR1 homeostasis in normal physiological conditions.

Complement receptor (CR2), a membrane glycoprotein (derived from a 110-120 kDa a non-glycosylated precursor), is one of a number of cell-surface proteins which bind activation and processing fragments of the complement system. Human CR2 is the receptor for the C3d/C3b, in addition to Epstein Bar Virus, CEBL, and CD21 and are expressed in all mature B – lymphocytes, follicular dendrite cells as well as on T lymphocytes (Fischer et al., 1991). CR2 ligands include complement C3d and Epstein-Barr virus glycoprotein 350/220. CR2 on B lymphocytes plays on antigen dependant enhancing role in the immune response by triggering the proliferation of pre activated cells and augmenting calcium signals induced in response to cross linking of sig M. (Cooper et al.,1976; Carter et al., 1988). Monoclonal antibodies have been developed and used to probe the structural correlates of CR2 functions. CR2 has been molecularly cloned and its primary amino acid sequence deduced. These data indicate that it shares characteristic structural features with a number of other complement and non-complement cell membrane and plasma proteins. Several of the complement-associated
proteins in this family possess regulatory functions; they are encoded by linked genes which have been localized to band q32 on chromosome 1. CR2 has been expressed in primate and rodent cells by transfection of cDNA in antigenically and functionally intact form. Functional role of CR2 on T cells is not clear. Recent evidence emphasizes the potential role of complement receptor on T cells in the regulation of immune response and infection with lymphocytotropic viruses. Also studies have shown that the serum soluble complement receptor 2 (CR2) is decreased in patients with RA (Masilamani et al., 2004). CR2 is also essential for the attachment of Epstein–Barr Virus (EBV) to the surface of B-lymphocytes in an interaction mediated by the viral envelope glycoprotein gp350 (Karen and Karlson, 2006).

Since in addition to CR1, CR3 and CR4 are also involved in the clearance of the immune complex and apoptotic bodies, and CR2 has a distinct role in the induction of B cell responses and their collaborative role in immunoregulation and immunoactivation is increasingly being realized, a comprehensive study on all these receptors in the understanding of the molecular mechanism underlying the autoimmune disorders, their diagnosis, prognosis and risk assessment are envisaged. The expression of CR2 on B cells may emerge as an additional laboratory tool in the assessment of rheumatoid arthritis activity. Both CR1 and CR2 are expressed by mature B cells (Wilson et al., 1986). The mechanism of complement regulation of the B cell response appears to involve the interaction of the complement activation products (C3b and C3d) and CR1 and CR2 on the cell surfaces, increasingly the retention of antigen in lymphoid tissue and enhancing the B cell response. Under many conditions, CR1 and CR2 levels on B-lymphocytes as well as CR1 on leucocytes and erythrocytes are reduced. In fact, soluble CR2 is found in the circulation in normal individuals and at increased levels in certain diseases (Masilamani et al., 2004). CR2 can be shed and or reduced in copy number on B or T cells and one or more undefined proteases may cut CR2 (Illges et al., 2000). In antibody responses, complement receptors 1 and 2 (CR1/2), expressed on B cells and follicular dendritic cells (FDCs) in mice, plays a central role. Complement-activating IgM administered with the antigen it is specific for, enhances the antibody response to this antigen (Rutemark et al., 2012). Complement receptors CR1 and CR2, which recognize activated products of C3 and C4, are dominantly expressed on B-cells and follicular dendritic cells. They are the principal receptors for uptake and retention of immune complexes. In their absence, memory B- cell survival is markedly impaired. This is likely because of the lack of antigen but could also reflect a role for complement C3d ligand (Roozendaal and Carroll, 2007). One of the recent studies suggests that CR1 and CR2 on
the surface of the B-lymphocytes collaborate to internalize C3b and iC3b proteins (Grattone et al., 1999). The involvement of B cells, complement activation and subsequent immune complex deposition has all been implicated in the pathogenesis of RA (Kremlitzka et al., 2012). The expression of the complement receptors 1 and 2 plays an important role in the pathophysiology of autoimmune disorder. The expression of these receptors may be different in different blood cells in health and disease. Few studies have been carried out on complement receptor 1; however, studies on complement receptor 2 are inadequate. No study had so far been carried out to elucidate the relationship of leucocyte CR1 and CR2 with the pathophysiology and disease activity of Rheumatoid Arthritis. Thus studies about complement receptor 1 and 2 are needed.

The present proposal, the first of its kind would provide baseline data as the expression levels of CR1 and CR2 on the lymphocytes, monocytes and neutrophils in the healthy individuals and its relationship with the pathophysiology and clinical disease activity of Rheumatoid Arthritis. The study thus would facilitate the evaluation of CR1 and CR2 disease activity markers for Rheumatoid Arthritis. This study therefore aimed at studying the expression of Leucocyte Complement Receptor 1 and 2, its modulation and significance in the pathophysiology of RA. The objectives were to:

a) Study and compare the expression profiles of the complement receptors CR1 and CR2 in three different blood cells i.e. lymphocytes, monocytes and neutrophils at the mRNA and protein levels in controls and patients suffering from Rheumatoid Arthritis.

b) Study the levels of C3, C3d and CIC in stored serum and plasma samples; relate that with the cell surface CR1 and CR2.

c) Evaluate the correlation among the above parameters and its relationship with the clinical disease activity.

1.1. Experimental Design

The study commenced with the standardization of various methods and techniques used in this investigation. The protocol for semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) was standardized to determine the levels of CR1 and CR2 transcripts. Flow cytometry was standardized to determine the levels of CR1 and CR2 protein expression and its distribution on total leucocytes. Rest of the methods/techniques namely for C3, C3d and CIC followed the protocols standardized in the lab earlier with minor modifications.
The experiments included: determination and comparison of the levels of CR1 and CR2 transcripts and proteins in patients and controls; the same was correlated with the clinical immunology laboratory parameters like levels of C3, C3d and CIC. The relationship of PBMCs (Peripheral Blood Mononuclear Cells) and Neutrophil -CR1 and CR2 transcript levels with the disease activity (DAS 28 score) was studied. A longitudinal follow up study was conducted to ascertain the role of CR1 and CR2 transcript and protein levels in active disease and remission and also to assess the prognostic significance of these transcripts. Significance of the results was evaluated by the statistical methods. The methods included Mann Whitney test, Students t-test, Spearman rho analysis, Two-tailed test and Paired t- test. Essentially, the experiments were designed and executed to achieve the aim and objectives of the investigation. One hundred controls and One hundred patients were enrolled for the study. Most of the studies involved all these subjects until/ otherwise specified. The study produced some interesting findings to suggest the role of the complement receptor 1 and 2 in RA disease pathology.
2. REVIEW OF LITERATURE

2.1 Rheumatoid Arthritis

Rheumatoid Arthritis (RA) is a chronic, progressive, systemic autoimmune disorder (Klippel et al., 2008). The disease affects multiple organs, including the joints, skin, heart, lungs and eyes. However, the primary target of the abnormal autoimmune response is the synovial joints (Coico and Sunshine, 2009).

RA is also the most common form of inflammatory arthritis in adults, affecting approximately 0.3 - 1.2% of world’s populations (Carmona et al., 2002); despite intensive investigations the etiology of RA remains undefined. Patients with RA complain of articular, systemic and extraarticular symptoms (Imboden et al., 2007). The most common articular symptoms are pain, swelling and joint stiffness. Common systemic features include low grade fever, fatigue and anemia. Approximately 50% of patients also complain of extraarticular symptoms such as nodules on the skin as well as sjögren syndrome which includes painful dry eyes and dry mouth. Although the pathogenesis of RA remains obscure, research indicates that immune system dysfunction involving the T lymphocytes, B lymphocytes and cytokines results in inflammation. This causes the synovial membrane to become swollen and form a pannus (McCance and Huether, 2006). The formation of the pannus is associated with destruction of the cartilage and erosion of the bone.

Although RA is a progressive and incurable disease, there are effective treatments which may slow the progression of the disease and reduce the associated disability.

2.1.1 Historical aspects of RA

RA is not a new disease. For example, skeletons from 6500 BC have been identified that demonstrate signs of RA. Paintings completed in the 16th and 17th centuries depict subjects who appear to have the classic symmetric polyarthritis of RA (Aceves - Avila et al., 2001).

The earliest known written description of chronic synovitis and polyarthritis that could have been RA comes from an ancient Indian text called the Charaka Samhita. However, it was Sir Alfred Garrod who, in 1859, first used the term "rheumatoid arthritis" to refer to a disease that was "an inflammatory affection of the joints not unlike rheumatism in some of its characteristics (Garrod, 1859). In 1939, Eric Waaler observed the presence
of an agglutination activating factor in the blood of patients with RA (Koopman and Moreland, 2005). This correlation was confirmed by Rose and colleagues. Agglutination activating factor was later renamed rheumatoid factor (RF) in 1949. To this day, RF levels are used in the diagnostic assessment of patients with suspected RA (Klippel et al., 2008). In the 1930s, Philip Hench also noted that RA in pregnant women often went into remission (Rubin et al., 2007). This led Hench to hypothesize that hormones could play a role in RA symptoms and treatment. Later, Edward Kendell and Tadeus Reichstein collaborated to purify the hormone cortisone which has anti-inflammatory properties, for therapeutic use (Rubin et al., 2007; Tortora and Derrickson, 2009).

2.1.2 Epidemiology of RA

Incidence and Prevalence

The prevalence of RA is ~0.8% of the population (range 0.3 -2.1%); women are affected approximately three times more often than men. In India, the prevalence of rheumatoid arthritis (0.75%) is similar to that in the west (Malaviya et al., 1993; Mijiyawa, 1995). Incidence in United States is estimated as 25 per 100,000 persons for men and 54 per 100,000 persons for women (Harris et al., 2005). RA is responsible for an estimated 250,000 hospitalizations and 9 million physician visits each year (ACR update 2002). Its economic impact is magnified by the high level of functional impairment it causes: untreated 20 to 30 percent of persons with RA become permanently work-disabled within three years of diagnosis (Sokka, 2003).

The prevalence increases with age, and sex differences diminish in the older age group (Khurana et al., 2008). RA is seen through the world and affects all races. However, the incidence and severity seem to be less in rural sub-Saharan Africa and in Caribbean blacks. The onset is most frequent during the fourth and fifth decades of life, with 80% of all patients developing the disease between the ages of 35 and 50. The incidence of RA is more than six times greater in 60 to 64 year old women compared to 18 to 29 year old women (Gabriel et al., 1999).

Mortality

Patients with RA have an overall higher mortality rate (Goodson et al., 2005). The life expectancy of a patient with RA is reduced by 3 to 7 years. Premature mortality appears to be related to severe articular disease that can lead to cardiovascular, infectious, and gastrointestinal disorders.
2.1.3 Clinical Manifestation of RA

RA affects multiple organs, although the primary target of the autoimmune response is the synovial joint. The disease course for RA varies from patient to patient. Typically, RA presents with gradual onset of joint pain and joint swelling which occur over a period of weeks or months. However, in approximately 10% of cases, the disease will present with severe symptoms beginning rapidly.

The symptoms of RA can be divided into the following categories:

- articular
- systemic
- extraarticular

Articular manifestations of RA

Patients with RA complain of pain, joint swelling, fatigue, and morning stiffness. The pattern of the joint involvement is an important clue in making the diagnosis of RA. RA can affect any synovial joint. However, early in the disease, the joints affected are the small joints of the hand, wrist, and feet. As the disease progresses, larger joints, such as the knee, ankle, and elbow, may be involved (Imboden et al., 2007). The joints are affected in a symmetrical manner. In other words, the same joints on both sides of the body are affected. It is possible to have asymmetric joint symptoms, but this is considered an atypical presentation. Occasionally, RA will initially present with symptoms in only one joint.

Joint stiffness is another common symptom in RA. The stiffness, characteristically, is present in the morning and lasts for more than 1 hour. Patients may report that they need to wake up from bed 1 to 2 hours earlier than usual to loosen up. Some patients may take a warm shower in order to improve morning function (Klippel et al., 2008).

Systemic manifestations

Patients with RA experience a number of systemic symptoms (Wolfe et al., 2003, 2005). Low grade fever, fatigue, weight loss, and decreased appetite are some common systemic complaints.
Extraarticular manifestations

Although the joints are the primary targets of the inflammatory process, other structures, such as the skin, heart, lungs, and blood vessels, may also be affected by the abnormal immune response (Coico and Sunshine, 2009). The effects of RA on these other structures are considered extraarticular manifestations. Approximately 50% of patients report these extraarticular complaints. The following table (Table.1) highlights some common extraarticular manifestations of RA.

Table 1: Extraarticular Manifestations of RA

<table>
<thead>
<tr>
<th>Clinical Condition</th>
<th>Organ(s) Affected</th>
<th>Potential Manifestations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rheumatoid nodules</td>
<td>Skin</td>
<td>Firm lumps over pressure areas such as the elbows and fingers</td>
</tr>
<tr>
<td>Keratoconjunctivitis sicca</td>
<td>Eyes</td>
<td>Dry eyes</td>
</tr>
<tr>
<td>Felty syndrome</td>
<td>Blood and spleen</td>
<td>Decreased white blood cells, Decreased platelets, Enlarged spleen</td>
</tr>
<tr>
<td>Coronary artery disease</td>
<td>Heart</td>
<td>Coronary heart disease, angina, hypertension</td>
</tr>
<tr>
<td>Pericarditis</td>
<td>Heart</td>
<td>Scarring in the sac surrounding the heart that restricts the heart from filling with blood and pumping effectively</td>
</tr>
<tr>
<td>Pericardial effusion</td>
<td>Heart</td>
<td>Fluid in the sac surrounding the heart that may cause shortness of breath</td>
</tr>
<tr>
<td>Small vessel vasculitis</td>
<td>Blood vessels</td>
<td>Infarction of finger tips</td>
</tr>
<tr>
<td>Pulmonary(pleural) effusion</td>
<td>Lungs</td>
<td>Collection of fluid around lungs that may cause shortness of breath</td>
</tr>
<tr>
<td>Interstitial lung disease</td>
<td>Lungs</td>
<td>May lead to shortness of breath, May progress to a honeycomb appearance on X-ray</td>
</tr>
</tbody>
</table>

Source: Klippel et al., 2008
2.1.4 Pathogenesis of RA

Joint damage in rheumatoid arthritis begins with proliferation of synovial macrophages and fibroblasts after a triggering incident, possibly autoimmune or infectious. Lymphocytes infiltrate perivascular regions and endothelial cells proliferate. Neovascularization then occurs. Blood vessels in the affected joint become occluded with small clots or inflammatory cells. Over time, inflamed synovial tissue begins to grow irregularly, forming invasive pannus tissue. Pannus invades and destroys cartilage and bone. Multiple cytokines, interleukins, proteinases and growth factors are released, causing further joint destruction and the development of systemic complications (Firestein, 2005; Harrison, 2008). Despite intense scientific research, the cause of RA remains unclear. The following is a summary of some of the key steps in the pathogenesis of RA (McCance and Huether, 2006; Fauci et al., 2008):

- an environmental or infectious trigger initiates an abnormal autoimmune response in genetically susceptible individuals.
- this trigger leads to activation of T lymphocytes and B lymphocytes.
- an inflammatory response ensues, with production of cytokines, proliferation of osteoblasts, and synthesis of antibodies.
- this results in the formation of a pannus as well as destruction of the joint.
- the end result is the formation of a pannus that ultimately leads to damage to the joint.

T-cell autoimmunity

T lymphocytes have been identified in the synovium of people with RA, and are implicated in the pathogenesis of this illness. The RA synovium contains many inflammatory cells including dendritic cells, macrophages, lymphocytes, and plasma cells (Coico and Sunshine, 2009). However, T lymphocytes are the predominant cell type found in the synovium (Fauci et al., 2008).

Cytokines cause the lining of the blood vessels (vascular endothelium) to express more, such as TNF-α, adhesion molecules, such as intracellular adhesion molecule-1 (ICAM-1). The adhesion molecules on the blood vessels bind to adhesion molecules on the T cell. The T cell then moves from the blood vessel through the vascular endothelium to the
site of the inflammation (Doan et al., 2008). At the level of the inflamed synovial joint, T cells produce cytokines that activate macrophages and fibroblasts.

T cells must be activated before they can differentiate and participate in the immune responses (Tortora and Derrickson, 2009). Activation of the T cell requires an interaction among a T cell, an antigen, and a costimulator occurring at the same time. Adhesion molecules on the surface of the T cell and the APC also help activate the T cell by stabilizing the interaction between the two cells (Doan et al., 2008).

**Costimulatory pathways**

Two well-defined costimulatory molecules related to T-cell activation is CD80 and CD86. From this point forward, the nomenclature CD80/86 will be used to describe these two molecules. CD80/86 are expressed on the surface of macrophages, dendritic cells, and B lymphocytes. These costimulators bind to specific costimulator receptors on helper T cells. Helper T (T<sub>H</sub>) cells contain the costimulator receptors CD28 and CTLA-4 (Abbas et al., 2007). The CD28 and CTLA-4 receptors are similar in shape and bind to the same types of molecules. However, each receptor produces a distinct reaction in T cells:

- T cells are activated when CD28 binds with CD80/86
- T cells are suppressed when CTLA-4 binds with CD80/86
- CTLA4 binds with higher avidity to CD80/86 than CD28

**B-cell autoimmunity**

B cells are also implicated in the autoimmune response associated with RA. Activated helper T cells activate B cells through a series of signals (Larsen et al., 2006). This results in B cells differentiating into plasma cells and memory B cells. The plasma cells in turn produce autoantibodies.

In RA, some of these antibodies act against the patient's own tissues and are therefore considered autoantibodies. Autoantibodies have been found in the joints and blood of patients with RA. Many autoantibodies have been identified in RA patients. Rheumatoid factor (RF) and anti-cyclic citrullinated peptideantibody (anti-CCP), as well as antibodies directed against collagen, are particularly important (Imboden et al., 2007). Rheumatoid factor (RF) is an autoantibody against the Fc portion of the antibody immunoglobulin G (IgG). B cells in the synovium of patients with RA secrete RF. These B
cells produce 2 types of RF (IgG and IgM). Overall, RF is detected in the blood of 90% of patients with RA.

Anti-CCP is a key autoantibody associated with RA. These are autoantibodies that recognize and bind to citrulline (Koopman and Moreland, 2005). Citrulline is present in the inflamed joint. Anti-CCP is identified in 60% to 70% of patients at the time of diagnosis of RA. This test is 90% to 98% specific for the diagnosis.

Autoantibodies against collagen, an integral part of the synovial joint, have also been found in the synovium of patients with RA. In RA, a minority of patients have anticollagen antibodies that affect the complement system.

**Joint damage and deformities**

In the hand, RA usually spares the distal interphalangeal joint (DIP). However, the metacarpophalangeal (MCP) and proximal interphalangeal (PIP) joints are affected. The wrists and feet are also commonly affected by RA (Imboden et al., 2007). Schematic representations of the normal and rheumatoid joints were depicted in figure 1.

![Schematic Representation of Normal Joint and Rheumatoid Arthritis Joint](image)

**Figure 1:** *Schematic Representation of Normal Joint and Rheumatoid Arthritis Joint.*

*Source: Medicine Net.Inc.*

RA also results in partial dislocation, also known as subluxation, of the MCP joints and wrist. Dislocation occurs when both bones are not contained in the joint (McCance and Huether, 2006).
Joint deformities develop as a consequence of the damage caused by the inflammatory process. More than 10% of patients with RA will develop joint deformities in their hands within the first 2 years of the disease. At least one-third of patients with RA will develop some deformity during the course of their illness (Koopman and Moreland, 2005). Swan neck and boutonniere deformities occur in late RA (Imboden et al., 2007). The effects of RA include deformities in the wrists, hands, and fingers. Deformities of hand and foot joints in RA were depicted in figure 2.

**Figure 2:** Deformities of hand and foot joints in RA patient.
*Source: Medicine OPD, All India Institute of Medical Sciences, New Delhi, India.*

### 2.1.5 Diagnosis of RA

The classic symptoms of RA include joint pain, joint swelling, fatigue, and low grade fevers (Imboden et al., 2007). However, these same symptoms can be associated with conditions other than RA (Weinblatt and Kuritzky, 2007). The physician's challenge is to make an early and accurate diagnosis of RA. Joint erosions and joint deformities, once found on examination or x-ray, are largely irreversible. It is particularly important in RA to initiate appropriate treatment early in order to prevent joint damage and deformity as well as minimize disability. A physician's assessment of a patient may consist of the following elements (Koopman and Moreland, 2005):

- patient history
- physical examination
- laboratory tests
- imaging studies

**ACR criteria for classification of RA**

The American College of Rheumatology (ACR) has published criteria for the classification of RA, which can be used as an aid in making a diagnostic assessment
(Imboden et al., 2007). Caution must be exercised in this process, as the classification system was originally designed to be used in clinical trials, and not to make diagnostic decisions in individual patients. It should be noted that there is no single finding on a physical examination or abnormality on a laboratory test that will make the diagnosis of RA. In early-stage RA, there may be no clear evidence of joint damage on physical examination or imaging studies (Weinblatt and Kuritzky, 2007). Rather, the physician will need to make a diagnostic judgment based on all the available clinical data. A diagnosis of established RA depends predominantly on characteristic clinical features and the exclusion of other inflammatory processes. In 1987, the American College of Rheumatology developed revised criteria for the classification of RA. The following table (Table.2) summarizes the ACR classification.

**Table 2: 1987 ACR revised criteria for the classification of RA**

<table>
<thead>
<tr>
<th>Criterion</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morning stiffness</td>
<td>Morning stiffness in and around the joints, lasting at least 1 hour before maximal improvement</td>
</tr>
<tr>
<td>Arthritis of 3 or more joint areas</td>
<td>At least 3 joint areas simultaneously have had soft tissue swelling or fluid (not bony overgrowth alone) observed by a physician. The 14 possible areas are right or left PIP, MCP, wrist, elbow, knee, ankle, and metatarsophalangeal (MTP) joints</td>
</tr>
<tr>
<td>Arthritis of hand joints</td>
<td>At least one area swollen (as defined above) in a wrist, MCP, or PIP joint</td>
</tr>
<tr>
<td>Symmetric arthritis</td>
<td>Simultaneous involvement of the same joint areas (as defined in 2) on both sides of the body (bilateral involvement of PIP, MCP, or MTP is acceptable without absolute symmetry)</td>
</tr>
<tr>
<td>Rheumatoid nodules</td>
<td>Subcutaneous nodules, over bony prominences or extensor surfaces, or in juxtaarticular regions observed by a physician</td>
</tr>
<tr>
<td>Serum -rheumatoid factor</td>
<td>Demonstration of abnormal amounts of serum rheumatoid factor by any method for which the result has been positive in &lt;5% of normal control subjects</td>
</tr>
<tr>
<td>Radiographic changes</td>
<td>Radiographic changes typical of rheumatoid arthritis on posteroanterior hand and wrist radiographs, which must include erosions or unequivocal bony decalcification localized in or most marked adjacent to the involved joints (osteoarthritis changes alone do not qualify)</td>
</tr>
</tbody>
</table>

As defined by the ACR, a patient is diagnosed with RA if at least 4 of the 7 criteria are present. Criteria 1 to 4 must have been present for at least 6 weeks. Patients who have
been diagnosed with other diseases of the joints and bones can still be diagnosed with RA (Arnett et al., 1988).

**Assessment by physician**

The goal of the history and physical examination is to determine if the patient's symptoms are due to RA or if another diagnosis is more appropriate. In the history, the physician will try to elicit symptoms associated with RA, such as joint pain, stiffness, and swelling, as well as fatigue. The physician may inquire about the presence of extraarticular manifestations of RA such as eye pain, dry mouth, or skin lesions. The physical exam will include an evaluation to detect the following (Kwoh et al., 2002):

- joint swelling and tenderness
- joint range of movement
- increased temperature of skin
- crepitus with joint movement
- weakness and wasting of muscles
- rheumatoid skin nodules

**Laboratory tests**

In addition to a history and physical examination, a physician may order blood tests to confirm or exclude the diagnosis of RA. The following tests are commonly ordered:

- erythrocyte sedimentation rate (ESR)
- C-reactive protein (CRP)
- RF
- anti-CCP

The ESR and CRP, in conjunction with patient symptoms and physical examination, are used to assess disease activity. The CRP and ESR share the following features (Keogan et al., 2006):

- marker of inflammation
- usually elevated in RA and decline with treatment
- can be elevated in a number of other diseases
- increase in value suggests that RA is more aggressive
- return to normal values suggests that the RA is getting better with treatment.
In general, CRP values <0.2 mg/dL are considered normal and values >1 mg/dL are consistent with inflammation. There is a significant amount of variability in the normal values from laboratory to laboratory. Normal ESR values for:

- men are 0 mm/hour to 10 mm/hour
- women are 0 mm/hour to 15 mm/hour

However, the upper limit of normal increases with age and with obesity (Imboden et al., 2007).

RF is an autoantibody against the Fc region of the antibody IgG. Overall, RF is detected in the blood of 90% of patients with RA. It should be recognized that people with other diseases, such as cancer, infections, and other rheumatic diseases, may have an elevated RF. Up to 4% of healthy individuals will have an elevated RF (Klippel et al., 2008).

Anti-CCP (anti-cyclic citrullinated peptide) is a key autoantibody associated with RA. Anti-CCP autoantibodies recognize and bind to citrulline. Citrulline is produced when the synovium of a joint becomes inflamed. Anti-CCP antibodies are identified in 60% to 70% of patients at the time of diagnosis of RA. This test is 90% to 98% specific for the diagnosis (Imboden et al., 2007). The first-generation test to determine the presence of anti-cyclic citrullinated peptides was termed anti-CCPI. Anti-CCP2 is a second-generation test, and is the most widely used assay. Anti-CCP2 has slightly better performance characteristics than the older assay (Niewold et al., 2007). Specifically, anti-CCP2 may have a slightly higher sensitivity rate while maintaining the specificity of anti-CCPI (ACR update 2009).

Patients with RA who are positive for anti-CCP are more likely to have more active disease, with a greater number of swollen joints. In addition, patients with a positive test are more likely to have radiological progression of their disease (Niewold et al., 2007). There is a correlation between patients who smoke cigarettes and a positive anti-CCP test. This is of interest as smoking is a risk factor for RA and is associated with increased disease activity. A Swedish study showed that smoking is associated with elevations in levels of anti-CCP antibody (Klareskog et al., 2006).

**Imaging studies**

Imaging studies are useful in making the diagnosis of RA. These tests can also be employed to monitor the progress of the disease and determine if the prescribed
treatment is effective. When evaluating a patient, the first imaging study should be plain x-rays. If additional diagnostic studies are required, a magnetic resonance imaging (MRI) study is usually requested (Rindfleisch and Muller, 2005).

**Plain x-rays**

In RA, the earliest abnormality on x-ray would be osteopenia, or thinning of the bones around the joint. However, this abnormality is not specific for RA. As RA progresses, erosions of the bone and joint space narrowing (JSN) will become apparent. Bone erosions and JSN can first present within 6 to 12 months of the initial presentation of RA. As the disease progresses, x-rays may demonstrate abnormalities such as joint misalignment and joint dislocation (Klippel et al., 2008). Plain film or standard x-rays of the hands, wrists, and feet are commonly ordered at the time of initial diagnosis and are repeated periodically. X-rays of these joints are preferred, as opposed to studies of larger joints, because they are thinner than other joints in the body, and therefore more likely to demonstrate the classic joint erosions associated with RA. Radiographic evidence of RA is apparent within the first 2 years of the disorder in 30% to 60% of patients. Radiographic evidence of damage occurs in almost 99% of patients who are both positive for RF and followed for >5 years (Koopman and Moreland, 2005).

**MRI, ultrasound, and CT scanning**

MRI and ultrasound are imaging modalities that are more sensitive methods for detecting joint erosions and visualizing the synovium. MRI can detect bone erosions within 4-months of the onset of RA. This is before abnormalities would become apparent on plain x-rays (Weinblatt and Kuritzky, 2007).

**Computed tomography (CT) scanning** can be helpful in situations where MRI cannot be performed. For example, an MRI cannot be performed in patients with a heart pacemaker because the MRI device can cause malfunction of the pacemaker (Klippel et al., 2008).

**2010 rheumatoid arthritis classification criteria**

An American college of Rheumatology/European League Against Rheumatism collaborative initiative replaces existing ACR criteria published in 1987, which focused on established, rather than early indicators of disease. The new 2010 criteria focus on early diagnosis and treatment, which has become increasingly important with the advent of more effective drug treatments over the past decade. The new criteria classify
“definite RA” as: signs and symptoms of RA, to decrease disability and to slow progression of articular damage assessed radiographically.

The major side effects are injection site reactions. It is rarely effective in those who have failed TNF-neutralizing therapy (Oslen and Stein, 2004). Rituximab, a chimeric antibody directed to CD20 that depletes mature B cells, has been approved for treatment of RA patients who have failed anti-TNF therapy. The major adverse events relate to transfusion reactions that can be controlled with glucocorticoids. Abatacept is a fusion protein consisting of CTLA4 and Fc protein of IgG1. It inhibits T cell activation by competitively inhibiting the co-stimulation of T cells that result from interaction of T cell- expressed CD28 and CD80/86 expressed by antigen presenting cells. It is usually reserved for patients who have failed TNF-neutralizing therapy or in those who have contraindications to TNF blockade. Abatacept is tolerated quite well (Maini et al., 1999; Barton and Ollier, 2002; Moreland, 2004). The immunosuppressive drugs azathioprine, leflunomide, cyclosporine and cyclophosphamide have been shown to be effective in the treatment of RA and to exert therapeutic effects similar to those of the DMARDs. They cause a variety of toxic side effects. Therefore, these drugs have been reserved for patients who have clearly failed therapy with DMARDs and biologics (Sanders and Harisdangkul, 2002). Surgery plays a role in the management of patients with severely damaged joints.

2.1.6 Complications related to RA

Female sex, a positive family history, older age, silicate exposure and smoking are associated with an increased risk for developing rheumatoid arthritis (Harris, 1990; Kuder et al., 2002; Firestein, 2005). Consumption of more than three cups of coffee daily- particularly decaffeinated coffee also may contribute (Mikuls et al., 2002) and oral contraceptives use (Kuder et al., 2002) are associated with decreased risk. Three in four women with rheumatoid arthritis experience significant improvement in symptoms when pregnant, usually with a recurrence after delivery (Harris et al., 2005). Patients with RA are at a greater risk to suffer from the following conditions (Imboden et al., 2007):

- cardiovascular disease
- osteoporosis
- infections
- certain cancers, including lymphomas
• carpal tunnel syndrome
• spinal cord injury of the cervical spine
• synovial cysts

Scales to assess disease activity and progress in RA

In everyday clinical practice, physician may use the overall tender or joint count as a method of determining disease activity. The progression or remission of RA can also be assessed by a measurement tool that incorporates a number of clinical parameters.

• ACR 20
• Disability Activity Score (DAS)
• Disability Activity Score 28 (DAS28)
• Sharp-Genant and Sharp-van der Heijde Radiological Scores
• Stanford Health Assessment Questionnaire (HAQ)
• Visual Analog Scale (VAS) for pain
• Fatigue scales

ACR measures of disease activity and ACR 20

The ACR has developed criteria to define improvement in RA. This assessment is based on physician evaluation, patient self-reporting, and laboratory data. The ACR criteria outline 7 measures of disease activity that should be evaluated in a patient with RA (Felson et al., 1995).

In clinical trials, the patient must achieve at least 20% improvement in the following areas: tender joint count and swollen joint count and 3 of the 5 core ACR measures, which are

• patient pain assessment
• patient global assessment
• physician global assessment
• patient self-assessed physical disability
• acute phase reactants (ESR or CRP)

DAS, DAS28 and EULAR response criteria

The Disability Activity Score (DAS) and Disability Activity Score 28 (DAS28) are also measures of disease activity (Fransen and van Riel, 2005). The DAS and DAS28 include a
count of the number of tender and swollen joints as well as the results of laboratory tests. Both the DAS and DAS28 have an optional assessment of the general health status of the patient. The following table (Table 3.3) summarizes some of the key features of the ACR, DAS, and DAS28.

Table 3: Measures of Disease Activity

<table>
<thead>
<tr>
<th>Disease Activity Measure</th>
<th>ACR Definition of DAS Improvement in RA</th>
<th>DAS</th>
<th>DAS28 (updated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tender joint count</td>
<td>&gt;28</td>
<td>53</td>
<td>28</td>
</tr>
<tr>
<td>Swollen joint count</td>
<td>&gt;28</td>
<td>44</td>
<td>28</td>
</tr>
<tr>
<td>Patient’s assessment of pain</td>
<td>VAS or questionnaire</td>
<td>Not included</td>
<td>Not included</td>
</tr>
<tr>
<td>Patient’s global assessment of disease activity</td>
<td>VAS or questionnaire</td>
<td>Not included</td>
<td>Not included</td>
</tr>
<tr>
<td>Physician's global assessment</td>
<td>VAS or questionnaire</td>
<td>General health assessment on a visual scale (optional)</td>
<td>General health assessment on a visual scale (optional)</td>
</tr>
<tr>
<td>Patient’s assessment of physical function</td>
<td>Questionnaires focusing on health or well-being</td>
<td>Not included</td>
<td>Not included</td>
</tr>
<tr>
<td>Acute-phase reactant level</td>
<td>ESR or CRP level</td>
<td>ESR or CRP level</td>
<td>ESR or CRP level</td>
</tr>
</tbody>
</table>

*Source: Felson, 1995*

There are similarities and differences between the ACR tool and the DAS and DAS28. Specifically, all 3 of the assessment tools include a count of the number of swollen and tender joints as well as the results of blood tests. The DAS and DAS28 contain an optional assessment of the patient's general health. In contrast, there are no optional components to the ACR evaluation tool. The DAS score has values that range from 0 to 10. The DAS28 score has values that range from 0 to 9.4. The European League Against Rheumatism (EULAR) has developed a method of transforming the DAS and DAS28...
scores so that the disease activity can be categorized as high, moderate, or low (Fransen et al., 2003). The following table (Table 4) summarizes the cut-off points for the DAS and DAS28 scoring system.

### Table 4: Cut-off Points for Disease Activity

<table>
<thead>
<tr>
<th>Low Disease Activity</th>
<th>Moderate Disease Activity</th>
<th>High Disease Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAS</td>
<td>≤ 2.4</td>
<td>2.4 &lt; DAS ≤ 3.7</td>
</tr>
<tr>
<td>DAS28</td>
<td>≤ 3.2</td>
<td>3.2 &lt; DAS28 ≤ 5.1</td>
</tr>
</tbody>
</table>

*Source: Fransen et al., 2003*

A patient with a DAS28 score of <2.6 is considered in remission. This is the only scale available where remission is defined. ACR does not have a definition for remission. Although the DAS and DAS28 contain fewer criteria, they correlate positively with ACR scores of RA disease activity (Fransen and van Riel, 2005).

#### 2.1.7 Etiology of RA

Although the specific cause of RA is unknown, there are some theories about its origin. There are a number of factors implicated in the onset and disease course of RA such as:

- genetic factors
- hormonal factors
- infectious factors
- environmental and dietary factors

#### Genetic factors

There appears to be a genetic predisposition for RA. The risk of developing RA is greater in identical twins when compared to fraternal twins. Approximately 15% to 20% of affected identical twins both have RA (Fauci et al., 2008). A person's risk of developing RA is also increased by 50% if a family member has been diagnosed with RA (Klippel et al., 2008). Genetic variations of genes on a specific area of the sixth chromosome are...
associated with the development of RA and other rheumatic diseases. Collectively, these genes are called the human leucocyte antigen (HLA) or major histocompatibility complex (MHC).

The terms HLA and MHC are used interchangeably (Tortora and Derrickson, 2009). The HLA genes code for many proteins related to the inflammatory response. There are at least 10 alleles on the MHC that are implicated in the development of RA. The term shared epitope denotes that many of these alleles have a similar sequence of amino acids. The shared epitope hypothesis is a theoretical framework that tries to identify a common molecular structure to understand how T cells affect the immune response in RA (Winchester, 2006). The following table (Table.5) provides some examples of genetic variability associated with RA.

Table 5: Genetic Variability in RA

<table>
<thead>
<tr>
<th>Allele</th>
<th>Community With Increased Risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-DRB-4</td>
<td>Western European populations</td>
</tr>
<tr>
<td>HLA-DR-1</td>
<td>Spanish, Basque, and Israeli populations</td>
</tr>
<tr>
<td>PADI</td>
<td>Japanese populations</td>
</tr>
</tbody>
</table>

Source: Suzuki, 2003

However, genetics account for 60% of the determining factors in the development of RA. Therefore the identification of new genes associated with RA susceptibility and clinical features is an important challenge.

**Hormonal factors**

The incidence of RA is higher in women (Helmick et al., 2008). Furthermore; pregnancy affects the clinical course of RA. Specifically, 75% of women with RA go into spontaneous remission during pregnancy. During pregnancy, there is an increase in the levels of hormones such as estrogen and progesterone (Tortora and Derrickson, 2009). However, after delivery, 90% of patients will have an exacerbation of RA symptoms. These observations suggest that hormonal factors may play a role in the development and clinical course of RA. Hormones can affect T cells and B cells. For example, hormones may cause the development of certain T cells to produce cytokines; this could potentially enhance or suppress the immune response. Furthermore, the detrimental role of estrogen in RA could be related to its effect on decreasing apoptosis in certain populations of B cells. This would result in an increase in B cells that act against the
body's own tissues (Klippel et al., 2008).

**Infectious factors**

Bacteria and viruses have been implicated in the pathology of RA (Klippel et al., 2008). It is hypothesized that an infectious agent could trigger the innate immune system and subsequently activates the adaptive immune system. One virus that may be involved in the development of RA is the Epstein-Barr virus. This virus activates 8 lymphocytes that result in increased production of rheumatoid factor. The following are some other infectious agents that may be involved in RA (Fauci et al., 2008):

- mycoplasma
- cytomegalovirus
- paravirus
- rubella virus

However, no definitive link has been identified between RA and an infectious pathogen.

**Environmental and dietary factors**

Cigarette smoking is a risk factor for RA (Koopman and Moreland, 2005). RA patients who smoke appear to have a worse clinical course. It is hypothesized that inhaled smoke may articulate the immune response in people who are genetically susceptible to RA (Klippel et al., 2008). Nutritional factors have also been implicated in RA (Mikulus et al., 2002). For example, Inuit people in Greenland consume food with n-3 fatty acids and appear to have a low risk for developing autoimmune disorders, such as RA (Galarraga et al., 2008).
2.2 Complement System

Complement was discovered more than a century ago (Ehrnthaller et al., 2011) as a heat labile component of blood plasma, which conferred bactericidal properties and complemented the activity of antibodies (Ab) to lyse red blood cells (RBCs) from other species. It consists of more than 30 proteins consisting of plasma proteins in the form of inactive enzyme precursors, soluble or cell-bound regulatory proteins and cell membrane receptors specific for complement protein cleavage fragments. It has a unique ability to discriminate “non-self” structures from “self” structures. Its primary function is to destroy and remove the foreign substances, microorganisms and apoptotic cell debris either by direct lysis or by mediating leucocyte function in inflammation and innate immunity (Ehrnthaller et al., 2011). Activation of the zymogens by pathogen, opsonized or un-opsonized by antibody leads to the generation of active peptides. These peptides affect a range of responses pivotal in the destruction and clearance of pathogens. Exaggerated or uncontrolled activation of the complement system leads to increased generation of inflammatory peptides. This in turn contributes significantly to the pathophysiology of immuno-inflammatory disorders.

2.2.1 Complement activation pathways

The complement cascade consists of three separate pathways that converge in a final common pathway. The phases of activation are initiation, amplification and formation of terminal complement complex. The mode of initiation is different for each of the pathways, amplification follows a common scheme and formation of terminal complement complex is the event where all three pathways cause the formation of membrane attack complex. The pathways are the antibody-dependent classical pathway (C1qrs, C2, C4), the alternative pathway (C3, factor B, properdin), and the mannan-binding lectin [MBL] pathway. The three main pathways of complement activation converge at C3. However, C3-independent pathways can activate C5 and other downstream complement components during IgG-initiated inflammatory responses (Auger et al., 2012). Schematic representation of the complement system is depicted in figure 3.

The Classical Pathway (CP)

The classical pathway is initiated by antigen-antibody complexes (Figure 3). The classical activation pathway involves proteins C1q, C1r, C1s, C2, C4 and C3 (Kishore and Reid, 2000). The C1 component consists of three subunits: C1q, C1r and C1s. C1q contain
six combining sites that are receptors for a specific sequence found on the Fc portion of IgG1, IgG2, IgG3 and IgM. These immunoglobulins, when complexed to antigen, will bind to C1q and cause activation of the molecule. Activated C1q generates an enzymatic activity in C1r that cleaves C1s and thereby generates the first major enzyme in the complement system, C1 esterase.

![Figure 3: Overview of the Complement system. The complement cascade consists of classical, lectin and alternative pathways that converge in a final common pathway. Source: Nilsson et al., 1990.](image)

C1 esterase cleaves C4 and C2, whose major cleavage products forms the bimolecular complex, C4b2a, which has C3 convertase enzymatic activity. The C3 convertase cleaves C3 and generates a small fragment, C3a, and a larger fragment C3b. Attachment of C3b to C4b2a complex forms the C4b2a3b complex, which has C5 convertase activity. Once the C5 convertase is generated, the membrane attack complex is activated. CP can also become activated by complexes containing chromatin or C-reactive protein or by surface blebs of apoptotic cells. Heparin (a polyanionic anticoagulant) and protamine (a polycation that is used to block heparin) when present in equimolar concentrations, can activate the classical pathway. Various other polyanions (eg. DNA and RNA) are thought
to be able to react directly with C1q to activate the classical pathway (Jacubik et al., 2000).

Alternative pathway (AP)

The alternative pathway provides a nonspecific natural defense system against microorganisms and other pathogens, which operate independently of specific antibody (Pangburn et al., 1983). It is known that the activation occurs on the surface of certain fungi, bacteria, lymphoblastoid cells and virus infected mammalian cells that have repetitive polysaccharides or lipopolysaccharide structures and an absence of sialic acid. First mechanism is “tick over”, which initiates alternative pathway by hydrolysis of the intra-molecular thioester bond of C3, including a conformational change in C3 and formation of C3i (Pangburn et al., 1983; Pangburn and Muller-Eberhard, 1984). C3 is continuously auto activated at a low level in plasma and undergoes slow and spontaneous hydrolysis to C3 (H$_2$O), which is not able to bind to surface, but is functionally equivalent to C3b. C3 (H$_2$O) binds factor B in the presence of Mg$^{2+}$. Factor B bound to C3 (H$_2$O) is cleaved by factor D releasing fragments Ba, which may have mitogenic activity. The spontaneously generated convertase C3 (H$_2$O) Bb cleaves C3 releasing the anaphylatoxins C3a and exposing the binding site on C3b. C3b nonspecifically binds to any surface of host cells and pathogenic organisms alike.

The other mechanism is the direct attack on the thioester bond of C3 by a hydroxyl group on a surface other than water resulting in covalent binding of C3 to the surface antigen. Since C3bBb cleaves several C3 molecules into C3b subunits of new C3 convertases, an effective positive feedback amplification loop is generated (Pangburn et al., 1983; Pangburn and Muller-Eberhard, 1984). Once activation occurs, the generation of C3b is accelerated. The convertase of the two pathways are homologous to each other. C4 and C3 are homologous, so are C4b and C3b, C2b and Bb (Campbell et al., 1981). In view of these structural similarities, it is not surprising that the two enzyme complexes, C4b2b and C3bBb, split C3 in the same way.

The Mannan Binding Lectin Pathway

The Lectin pathway closely resembles the classical pathway. Instead of C1 the pathway is initiated by mannose binding lectin (MBL) that binds to carbohydrates containing mannose or N-acetyl glucosamine residues (Reid and Turner, 1994; Matsushita and Fujita, 1992). MBL belongs to a family of collectins and has structural homology to C1q. MBL binds to MBL-associated serine proteases MASP-1 and MASP-2 to form a C1-like
complex (Matsushita and Fujita, 1992). MBL binds to the target structure calcium-dependently, and the binding results in conformational changes and activation of the MASP. The cell bound complex cleaves C2 and C4 similarly as C1 of the CP. The rest of the cascade is identical to that of the CP. Thus, C3 is central and common link to these different C-activation pathways. Amplification of either of the pathways leads to the generation of C5b which then binds sequentially to C6-C9 components to form membrane attack complex (MAC) (Campbell et al., 1981).

The non-enzymatic components C6-C9 then bind sequentially to C5b. Binding of C6 to C5b induces a conformational change in C6 making it capable of binding C7. The C5b-7 complex is hydrophobic and capable of binding to lipid membranes. C8 in turn may bind either to a cell-bound or a soluble C5b-7 complex. C5b-8 binds C9 and when multiple C9 molecules polymerize and penetrate through the cell membrane, a cytolytic MAC is formed and the membrane is perforated. The TCC is a joint name for both the soluble and the membrane associated complexes (C5b-7, C5b-8, C5b-9, C5b-9n) (Muller-Eberhard, 1986). This complex causes osmotic lysis of the invading microorganisms.

### 2.2.2 Functions of the complement system

The most frequently encountered crisis in individuals deficient in the components of the complement system are recurrent and severe bacterial infection and immune complex disease indication that the primary function of the complement system is killing of invading bacteria and solubilising the immune complex (Morgan and Walport, 1991). Apart from these roles the complement system enhances inflammation at the sites of infection, influences the activity of numerous cells of the immune system, and helps in clearing apoptotic body from the circulation and assist in the induction of the antibody response. The c-system thus bridges the innate and adaptive immunity. The effects of the C system may involve the whole complement, systems or only individual components or fragments. The over-activation of the C system and the excessive consumption of C proteins in the effector phase of SLE are documented by hypocomplementia and increased C activation products like C3d in the circulation of SLE patients (Arora et al., 2004).

Activation of the complement cascade, with the formation of the effector MAC unit, results in a cytotoxic and cytolytic reaction and this process involves the whole complement cascade. Target cells for MAC action may be heterologous erythrocytes, autologous or foreign nucleated cells, bacteria (gram-negative), microscopic fungi,
viruses with a surface envelope and virus infected cells. The result of cytotoxic complement reaction is beneficial for the body when there is elimination of the infectious agent or damaged cells. However, cytotoxic complement reaction may be harmful when there is damage to autologous normal cells by immunopathological reactions.

Different fragments, released from components during complement activation, operate by a non-cytolytic mechanism through specific receptors present on various cell types, which lead to various biological functions. The direction, role and intensity of the biological responses depend on the affinity and density of receptors and on the function of cells bearing receptors. From the functional standpoint, complement receptors can be divided into two types: the receptors binding to small fragments of C3, C4 and C5. The first type of receptor mediate adherence of cells and other particles with bound C3b or C4b fragments and are named CR1 to CR4. Adherence reaction mediated through the CR receptors on phagocytes lead to stimulation of phagocytosis, activation of metabolism, enhancement of secretory function and movement of phagocytes into the site of inflammation. These receptors, when present on the non-phagocytic cells, are involved in a variety of immunoregulatory reactions. CR1 on erythrocytes binds circulating immune complexes optionized by complement fragments and transport them to the liver where the immune complexes are partially degraded. In addition to inducing phagocytosis, ligation of complement receptors on neutrophils, monocytes and macrophages may also stimulate exocytosis of granules containing powerful proteolytic enzymes, and free radical production through respiratory burst (Kajita and Hugli, 1990). The second group of receptors reacts with small complement fragments (C4a, C3a, C5a) as well as with C1q, Ba, Bb and factor H. the cleavage fragments C3a, C4a, C5a and probably C2b constitutes potent stimuli for the initiation of the local inflammatory response and are referred to as anaphylatoxins. Stimulation of the receptors by the anaphylatoxins results in various biological effects like chemotaxis, secretion of vasoactive amines, mediation of the inflammatory and anaphylactic reactions etc. The anaphylatoxins have powerful effects on blood vessel walls, causing contraction of smooth muscle and an increase in vascular permeability (Hugli et al., 1983). C5a is the most potent anaphylatoxin being approximately 100 times more effective than C3a, and 1000 times more effective than C4a.

Evidences in the literature suggest that the complement system can perform an important immunoregulatory function through its role in humoral immunity (Molina et
al., 1996), T cells immunity modulation (Kaya et al., 2001) and tolerance regulation for own nuclear antigens (Carroll, 2000).

2.2.3 Proteins of the cascade in disease

The C system is involved in the pathogenesis of many diseases. The complement system is implicated in human disease on two main fronts, namely, C deficiency and over-activation of C. Numerous disorders are a direct consequence of deficiency of one or more components of the C cascade (Rynes, 1982; Frank, 1987). In contrast, there are diseases that result from inflammation initiated or perpetuated by uncontrolled complement activation.

Complement pathway deficiencies:

Deficiencies of components of the complement system result in a wide variety of clinical presentations of disease, including recurrent bacterial infections, HAE, rheumatic disorders, leucocyte adhesion deficiency and HUS. With so many proteins involved, it is not surprising that inherited deficiencies (Ross et al., 1982; Figureueroa and Densen, 1991) of one or another are sometimes encountered in humans. The complement deficiency disorders are enlisted below.

- **C1q deficiency**: Individuals with C1q deficiency have markedly reduced levels of serum total hemolytic activity and C1 functional activity. This dysfunctional C1q is antigenically deficient when compared to normal C1q, does not bind to immunoglobulins. The most common clinical presentation of C1q deficiency is SLE (Pickering et al., 2000; Walport 2001, 2002). Some patients with C1q deficiency have sepsis and meningitis.

- **C1r and C1s deficiency**: Genetically determined deficiency of C1r and C1s is characterized by markedly reduced levels of C1r and moderately reduced levels of C1s. The clinical expression of these deficiencies includes SLE and glomerulonephritis (Pickering et al., 2000; Walport 2001, 2002).

- **C4 deficiency**: Patients with complete C4 deficiency have markedly reduced levels of both functional and antigenic C4 in their serum. Some patients with complete C4 deficiency have an increased susceptibility to sepsis and meningitis. C4 deficiency results from homozygous null alleles of both C4A and C4B gene loci (Blanchong et al., 2001).
- **C2 deficiency**: C2 deficiency is the most common homozygous complement deficiency occurring in 1:10,000 to 1:20,000 individuals (Figureueroa and Densen, 1991). This emphasizes the important role of the complement system in clearing away antigen-antibody complexes. A deficiency of C2 (or one of the other early components) is frequently found in patients with the autoimmune disorder, rheumatic disorders, SLE and discoid lupus.

- **C3 deficiency**: Patients with C3 deficiency have markedly reduced levels of antigenic and functional C3. These patients exhibit deficient serum hemolytic, opsonic, chemotactic and bactericidal activities. An inherited deficiency of C3 predisposes the person to frequent bouts of bacterial infections and susceptibility to rheumatic disease like RA and SLE (David et al., 2009).

- **C9 deficiency**: Most people who cannot make C9 have no more of a problem with bacterial infections than those who can. Laboratory studies suggest that the C5b-6-7-8 complex by itself is able to lyse bacteria although not as efficiently as C9 (Nagata et al., 1989).

- **C1INH deficiency**: A deficiency of C1INH produces hereditary angioneurotic edema (HANE). Patients are at risk of occasional explosive triggering of the complement system. The massive release of anaphylatoxin (C3a, C5a) may cause dangerous swelling (edema) of the airways, as well as of the skin and intestine.
2.2.4 Regulation and control of complement activation

Complement system has powerful cytolytic activity against which individual’s own cells (self-cells) should be protected as the complement proteins cannot differentiate between self and non-self antigens. A tight control of complement amplification is therefore, essential to prevent uncontrolled and rapid consumption of the complement components and the consequent inflammation in response to small stimuli (Figure 4).

**Figure 4:** Activation and regulation of the complement system. A tight control of complement amplification is essential to prevent uncontrolled and rapid consumption of the complement components and the consequent inflammation in response to small stimuli.

Source: www-ermm.cbcu.cam.ac.uk/03006288h.htm

Several proteins have evolved to control the extent of complement activation in fluid phase and surfaces of self cells. These fluid phase proteins include C1-inhibitor (C1-INH) (Davis, 1988), C4-binding protein (C4BP) (Campbell et al., 1981), factor H and
factor I. Some of the fluid phase proteins such as clustering inhibit the formation of cytolytic MAC.

These fluid phase proteins serve to limit the generation of complement fragments such as C4b and C3b and also render the generated fragments inactive thereby reducing the extent of cellular damage. C4b and C3b which have escaped inactivation by fluid phase inhibitors of complement activation and are already fixed to self cells, are inactivated by cell membrane regulators of complement such as DAF (Nicholson -Weller, 1982; Hourcade et al., 1989; Lublin and Atkinson, 1989), MCP (Hourcade et al., 1989; Liszewski et al., 1991) and complement receptor 1 (CR1) (Ahearn and Fearon, 1989; Hourcade et al., 1989; Wong, 2000). The complement cascade can be activated via classical pathway following an antigen-antibody reaction or via an alternative pathway which usually does not require an antibody as triggering factor. Both pathways converge at the level of C3 whose split products reflect C activation by either pathway. C3 is broken down by C3 convertases to a small C3a and a large C3b fragment. C3b is inactivated by C3b inhibitor (factor I) to form C3bi. A further trypsin-like enzymatic activity produces C3c and C3d fragments (Vergani et al., 1983).

Some of the cell membrane proteins, CD59 and homologous restriction factor (HRF), render MAC non-cytolytic while it is being formed on the self cell. Thus, fluid phase inhibitors in conjunction with membrane embedded inhibitors protect cells from autologous complement lysis. However, many pathogens do escape C-mediated cell lysis and immune attack by expressing CRPs on their cell surface by molecular mimicry. The acute-phase protein CRP recruits C1q to the surface of damaged cells and thereby initiates complement activation. However, CRP also recruits complement inhibitors, such as C4b-binding protein (C4bp) and factor H, which both block complement progression at the level of C3 and inhibits inflammation (Mihlan et al., 2011). The individual functions of these fluid phase and cell surface CRPs are summarized in Table 6.
### Table 6: Diverse Activities of Complement Regulatory Proteins

<table>
<thead>
<tr>
<th>Regulator</th>
<th>Ligands</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. Fluid phase serum proteins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C1 INH</td>
<td>C1r/C1s</td>
<td>Dissociates activated C1</td>
</tr>
<tr>
<td>C4 binding protein (C4BP)</td>
<td>C4b</td>
<td>Dissociates classical C3 convertase</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cofactor for Factor I</td>
</tr>
<tr>
<td>Factor H</td>
<td>C3b</td>
<td>Dissociates alternative C3 convertase</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cofactor for Factor I</td>
</tr>
<tr>
<td>Factor I</td>
<td>C3b/C4b</td>
<td>Degrades C4b and C3b (requires a cofactor)</td>
</tr>
<tr>
<td>Serum proteases</td>
<td>C5b67</td>
<td>Blocks fluid-phase MAC</td>
</tr>
<tr>
<td>S protein (vitronectin)</td>
<td>C5b67</td>
<td>Blocks fluid-phase MAC</td>
</tr>
<tr>
<td>Anaphylatoxin inactivator</td>
<td>C4a/C3a/C5a</td>
<td>Degrades C4a, C3a and C5a</td>
</tr>
<tr>
<td><strong>B. Integral membrane proteins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complement Receptor 1 (CR1, CD35)</td>
<td>C3b/C4b</td>
<td>Dissociates C3 convertase</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cofactor for Factor I</td>
</tr>
<tr>
<td>Decay Accelerating factor (DAF, CD55)</td>
<td>C3b/C4b</td>
<td>Dissociates C3 and C5 convertases</td>
</tr>
<tr>
<td>Membrane Cofactor Protein (MCP, CD46)</td>
<td>C3b/C4b</td>
<td>Cofactor for Factor I</td>
</tr>
<tr>
<td>Homologous restriction Factor (HRF, C8BP, MIP)</td>
<td>C8/C9</td>
<td>Prevents MAC insertion into lipid bilayer of autologous cells and lysis</td>
</tr>
<tr>
<td>MIRL (protectin, CD59)</td>
<td>C8</td>
<td>Blocks C7, C8 binding to C5b,6 preventing MAC formation and lysis</td>
</tr>
</tbody>
</table>

*Source: Zipfel and Skerka, 2009.*
2.3 Complement Receptors

The result of cytotoxic complement reaction may be beneficial (elimination of the infectious agent or damaged cells) or harmful (damage to autologous normal cells by immunopathological reactions) for the body. Different fragments, released during complement activation, operate by a non-cytolytic mechanism through specific receptors present on various cell types. The direction and intensity of the biological response depend on the state of the receptors (affinity and density) and on the function of cells bearing receptors. From the functional standpoint, complement receptors can be divided into two types:

**Adherent type receptors:** These receptors mediate adherence of cells and other particles with bound C3b or C4b fragments and are known as Complement receptors 1-4 (CR1 to CR4). Adherence reaction mediated through the CR receptors on phagocytes lead to stimulation of phagocytosis, activation of metabolism and secretory function and movement of phagocytes into the inflammatory site. CR1 is the receptor for the ligand C3b/C4b, CR2 (CR2, CD21) for C3d and CR3 (CR3, CD11b/18) for the ligand C3bi and CR4 (CR4, CD11c/18) for the ligand C3bi and C3dg. These receptors are discussed below.

**The other receptors:** The second group of receptors reacts with small complement fragments (C4a, C3a, C5a) as well as with C1q, Ba, Bb and factor H. Stimulation of these receptors results in various biological effects (chemotaxis, secretion of vasoactive amines, mediators of the inflammatory and anaphylactic reaction etc.).

- *Complement receptor 1 (CR1):* The receptor is expressed on all blood cells, kidney podocytes, follicular dendritic cells and astrocytes (Reynes et al., 1985). CR1 on erythrocytes binds to circulating immune complexes (that had activated complement) opsonized with C3b and transport them to the liver where the immune complexes are partially degraded and thus become more soluble (Walport and Davies, 1996). The receptor is discussed in detail in the later section.

- *Complement receptor 2 (CR2, CR3dg, CD21):* This receptor is expressed on B-lymphocytes, follicular dendritic cells, some epithelial cells, peripheral blood T cells. Complement receptor 2 (CR2/CD21) is an important receptor that amplifies B lymphocyte activation by bridging the innate and adaptive immune system. CR2 also play an important role in enhancing humoral immunity to T-dependent and T-independent foreign antigens (Carroll, 2000) and in regulating T-cell immunity.
to self and non-self antigens (Kaya et al., 2001; Pratt et al., 2002). The following table (Table. 7) summarizes complement receptors, their ligands and functions.

Table 7: Complement Receptors; Ligands and Functions

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Major ligand</th>
<th>Function</th>
<th>Cell Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR1</td>
<td>C3b/C4b</td>
<td>Immune adherence, immune complex clearance and regulation of C3b/C4b</td>
<td>Erythrocytes, macrophages, B cells, monocytes, polymorphonuclear leukocytes, FDC</td>
</tr>
<tr>
<td>CR2</td>
<td>C3dg/C3bi</td>
<td>B cell activation, immune complex localization, and EBV invasion into cell</td>
<td>B cells, FDC</td>
</tr>
<tr>
<td>CR3</td>
<td>C3bi</td>
<td>Phagocytosis</td>
<td>Macrophages, monocytes, polymorphonuclear leukocytes, FDC</td>
</tr>
<tr>
<td>CR4</td>
<td>C3bi</td>
<td>Phagocytosis</td>
<td>Macrophages, monocytes, polymorphonuclear leukocytes, dendritic cells</td>
</tr>
<tr>
<td>C3aR</td>
<td>C3a</td>
<td>Cell activation</td>
<td>Endothelial cells, mast cells, phagocytes</td>
</tr>
<tr>
<td>C5aR</td>
<td>C5a</td>
<td>Cell activation and chemotaxis</td>
<td>Endothelial cells, mast cells, phagocytes</td>
</tr>
</tbody>
</table>


CR2 ligands include complement C3d and Epstein-Barr virus glycoprotein 350/220. Regions of EBV have structural similarity to C3dg, which allows it to bind CR2, and thereby gain access to cell’s interior. It also acts as receptor for other components or activators of innate immunity such as IFN-α, an anti-viral cytokine and DNA-DNA containing complexes such as chromatin.

The binding of CR2 to IFN-α is speculated to cause B cell activation but their roles are still not clear. It is however, speculated that the binding to these ligands may be important in the development of SLE, characterized by enhanced IFN-α levels and loss of tolerance to DNA-containing self-antigens (Szakonyi et al., 2001).

- Complement receptor type 3 (CR3, C3bi receptor, CD 11b-18): The integrin, CR3, is a heterodimer that consist of 16 5kDa alpha chain non-covalently attached to a
95kDa beta chain (Kuby, 1997). The alpha chain has a binding site for the breakdown products of C3 (particularly C3bi, but also CR3b and C3dg). Most infectious particles become covered with various combinations of C3b, C3bi and C3dg. CR3 is particularly effective in triggering phagocytosis of these portal particles. Additionally CR3 has a lectin-binding capacity that allows it to bind specific carbohydrates on the surface of microorganisms. CR3 on neutrophils and monocytes may promote adhesion to modified endothelial cells. This facilitates the accumulation of inflammatory cells in an area of tissue injury.

- **Complement receptor type 4 (CR4, CD11c-18):** CR4, previously known as p150, 95, is also integrin. The alpha chain is 150 kDa while the beta chain is identical to that of CR3. CR4 is not only present on the same cell type as CR3 but also has similar ligand specificity (Kuby, 1997).

### 2.3.1 Complement Receptor 1 (CR1, CD35)

In 1953, RA Nelson initiated CR1 research, when he described binding reactions between human erythrocytes and specifically opsonized trypanemes and pneumococci, and coined the term ‘immune adherence’ receptor to describe this reaction. This immune adherence receptor of the RBC was shown subsequently to be specific for C3b in the immune complex. Detailed biochemical characterization of CR1 began with Fearon's purification of a 205,000 daltons mol. wt. glycoprotein from human RBC’s (Fearon, 1980).

In normal physiological conditions, exaggerated C- activation is kept under control by effective functioning of a number of CRPs. CR1; the human C3b/C4b receptor (CR1, CD35) has gained much attention. Reasons are manifold (Katyal et al., 2001). It is the best characterized receptor for the activated form of complement protein C3, C3b (Ahearn and Fearon, 1989; Delibrias et al., 1992). Binding with lower affinity was also demonstrated for the degraded form of C3b, C3bi, and for complement proteins C4b, C1q and mannan binding lectins (Tas et al., 1999; Ghiran et al. 2000). CR1 is a polymorphic transmembrane glycoprotein present on the surface of erythrocytes, B-cells, polymorphonuclear neutrophils, monocytes, macrophages, glomerular podocytes and some T-cells (Katyal et al., 2003). Erythrocyte CR1 is the chief vehicle for the clearance of circulating immune complexes and inhibits complement activation (Katyal et al., 2004). Various ligand with their binding affinities for CR1 are listed in Table 8.
Table 8: Various Ligands with their Binding Affinities for CR1

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3b</td>
<td>C3b is a primary ligand. CD 35’s affinity is greater for C3b than for C4b, weaker affinity is seen with iC3b and C3dg. In vitro, affinities are enhanced by reducing the ionic strength.</td>
</tr>
<tr>
<td>C4b</td>
<td>C4b is a primary natural ligand. CD35’s affinity is greater for C3b than for C4b, weaker affinity is seen with iC3b and C3dg. In vitro, affinities are enhanced by reducing the ionic strength.</td>
</tr>
<tr>
<td>iC3b</td>
<td>CD 35’s affinity is greater for C3b than for C4b, weaker affinity is seen with iC3b and C3dg. In vitro, affinities are enhanced by reducing the ionic strength.</td>
</tr>
<tr>
<td>C3dg</td>
<td>CD35’s affinity is greater for C3b than C4b, weaker affinity is seen with iC3b and C3dg. In vitro, affinities are enhanced by reducing the ionic strength.</td>
</tr>
<tr>
<td>iC3 (hemolytically inactive C3, C3(H2O), C3i)</td>
<td>Binds CD35 with affinity similar to C3b and C4b. In vitro, affinities are enhanced by reducing the ionic strength.</td>
</tr>
<tr>
<td>iC4 (hemolytically inactive C4, C4(H2O), C4i)</td>
<td>Binds CD35 with affinity similar to C3b and C4b. In vitro, affinities are enhanced by reducing the ionic strength.</td>
</tr>
</tbody>
</table>

Source: Lambris and Tsokos, 1986.

Structure and molecular biology of CR1

Human CR1 is a single chain, type 1 transmembrane glycoprotein composed of an extracellular domain of 1930 residues organized into 30 short repeats of a consensus element, anchored in plasma membrane through a hydrophobic transmembrane domain of 25 residues with a 43 residue cytoplasmic domain (Klickstein et al., 1987). It belongs to the family of proteins encoded by RCA gene cluster located on chromosome 1, q32. Other members of this family are the α-chain of the C4b-binding protein (C4bpα), factor H (fH), factor H–related proteins (FHR-1 through FHR-4), CR2, the CR1-like protein (CR1L), membrane cofactor protein (MCP, CD46), decay accelerating factor (DAF, CD55) (Rodriguez de Cordoba et al., 1985). The RCA gene cluster is depicted in Figure 5.

![Figure 5](https://www.nature.com/.../v6/n1/figure tab/6364150f1.html)
Structurally, the RCA proteins are similar in that they are predominantly composed of repeating motifs of approximately 60 to 70 amino acids, termed short consensus repeats (SCRs) (Rodriguez de Cordoba et al., 1985), or complement control protein repeats (CCPs). Each SCR has in common a number of highly conserved residues, including four invariant cysteines that form two disulfide bonds, imparting a unique double looped structure to the SCR.

**Molecular weight of CR1**

Four polymorphic forms have been identified with relative molecular weights of 160,000 (C), 190,000 (A), 220,000 (B) and 250,000 (D) kDa respectively controlled by 4 autosomal co-dominant alleles A, B, C and D (Dykman et al., 1985). Most common alleles of CR1 (A and B) having gene frequencies of 0.8 and 0.2. Rare alleles (C and D) occurred at a frequency of less than 5% (Moulds et al., 1996). The relative molecular weights of each allotype vary by 30 kDa if measured under non-reducing conditions. The molecular weight differences among the 4 allotypes persist even after treatment with endoglycosidase, indicating that the polymorphism is not the result of differential glycosylation (Wong et al., 1988).

The 220,000 Mr A allotype is composed of 30 SCRs, which can be arranged according to internal homology, beginning with the N-terminal SCR, into four tandem repeats of seven SCRs each (Klickstein et al., 1987). These repeats are termed LHR, with the most N-terminal LHR designated LHR-A, followed by LHR-B, LHR-C, and LHR-D (Figure 6).

![Figure 6](https://example.com/figure6.png)

**Figure 6:** Schematic representation of the most common size variant of CR1 and its functional domains. Extracellular Domain: 1930 residues, transmembrane Domain: 25 residues, Cytoplasmic Domain: 43 residues.

Source: nature.com/.../v6/n1/figure tab/6364150f1.html

The last two SCRs (SCR 29 and 30) do not fit into this homologous arrangement. Restriction map analysis of the 250,000 Mr CR1 B allele has indicated the presence of an additional LHR between LHR-A and LHR-B (Wong et al., 1988). Similar analysis of the
190,000 Mr CR1 C allele suggests that this allele is missing LHR-B, and thus consists of LHR-A, LHR-C, and LHR-D. Thus, the differences among the A, B, and C alleles of CR1 are due to the number of LHRs present, and suggest that these alleles arose from genetic recombination with unequal cross-over involving seven SCRs. This data also suggests that the D allele may contain a total of six LHRs, though this has yet to be demonstrated. The predicted structures of CR1 allotypes are shown in Table 9.

**Table 9: Molecular Weights of CR1 Allotypes**

<table>
<thead>
<tr>
<th>New name</th>
<th>Old name</th>
<th>CR1 size (Mr)b</th>
<th>CR1 size (Mr)c</th>
<th># of LHRs</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR*1</td>
<td>A or F</td>
<td>1,90,000</td>
<td>2,20,000</td>
<td>4</td>
<td>0.83</td>
</tr>
<tr>
<td>CR*2</td>
<td>B or S</td>
<td>2,20,000</td>
<td>2,50,000</td>
<td>5</td>
<td>0.15</td>
</tr>
<tr>
<td>CR*3</td>
<td>C or F'</td>
<td>1,60,000</td>
<td>1,90,000</td>
<td>3</td>
<td>0.01</td>
</tr>
<tr>
<td>CR*4</td>
<td>D</td>
<td>2,50,000</td>
<td>2,80,000</td>
<td>6</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

*Source: Wong et al., 1986; Katyal et al., 2001.*

In addition to these genetically controlled variations, tissue/cell-specific differences in N-glycosylation are responsible for minor size variations. Differences of 5 kDa in apparent molecular weight has been observed between CR1 isolated from erythrocytes and those isolated from neutrophils or T cells of the same individual (Lublin et al., 1988).

**Genetic polymorphisms of CR1**

CR1 gene is polymorphic. Both, the density and the structural polymorphisms of CR1 are known. The association of these polymorphisms with SLE and glomerulonephritis has been investigated. (Katyal et al., 2004) Results had been contradictory. (Kumar, 2002; Sivasankar et al., 2004) Majority of researchers believe that deficient expression of CR1 in disease is not inherited but an acquired phenomenon and polymorphisms have no role in the disease. Excessive cell surface proteolytic cleavage of CR1 is held responsible for this decline. Further our lab had brought the first experimental evidence on reduced CR1 mRNA transcript in the leucocytes of SLE patients (Arora et al., 2004). A simultaneous increase in the proteolytic cleavage of cell surface receptor was also evident.
Although an altered cytokine profile appears to modulate complement receptor expression in SLE, RA (Arora et al., 2000b) and other immune inflammatory disorders, effect of cytokines on complement receptors is poorly studied. It is of interest to study the role of immune complex, complement activation and therapeutics on the expression of CR1 and other complement receptors.

**Cellular expression of CR1**

In humans, CR1 is present on all human peripheral blood cells except platelets. It is expressed on variety of cells like erythrocytes (E-CR1), eosinophils, monocytes, macrophages, B-lymphocytes, a subpopulation of CD4+ T cells and polymorphonuclear neutrophils (PMN, N-CR1), (Ross et al., 1982; Weis et al., 1984; Reynes et al., 1985; Wilson et al., 1986). Distribution and function of CR1 on the surface of these cells are given in Table 7. CR1 is also found at several other sites including langerhan cells in the skin, kidney podocytes (G-CR1) follicular dendritic cells in lymphoid organs and astrocytes in the brain. CR1 is also present in plasma as the soluble form (sCR1) and in the urine as u-CR1. The following table (Table 10) shows the relative distribution and functions of cellular CR1.

**Table 10: Relative Distribution and Functions of Cellular CR1**

<table>
<thead>
<tr>
<th>Location of CR1</th>
<th>Average number of CR1/Cell</th>
<th>Functions on the surface of these cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythrocytes</td>
<td>500-600</td>
<td>Processing and transport of ICs</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>5000</td>
<td>Phagocytosis, Endocytosis of soluble ICs</td>
</tr>
<tr>
<td>Monocytes/macrophages</td>
<td>5000</td>
<td>Phagocytosis</td>
</tr>
<tr>
<td>B-Lymphocytes</td>
<td>20,000-40,000</td>
<td>Phagocytosis</td>
</tr>
<tr>
<td>T-Lymphocytes</td>
<td>Not known</td>
<td>Cell activation</td>
</tr>
<tr>
<td>Glomerular Podocytes</td>
<td>200,000</td>
<td>Trapping immune complexes</td>
</tr>
</tbody>
</table>


**Erythrocyte CR1 (E-CR1)**

The erythrocytes possess about 500 copies of CR1 per cell, while leucocytes have between 10 and 50,000 copies/cell. Since erythrocytes are nearly 1000 times more numerous than other vascular cells, they account for about 90% of CR1 in blood.
**E-CR1 density polymorphism:** CR1 copy number per erythrocyte is under the control of high (H) and low (L) copy number alleles whose frequencies are 0.73 and 0.27 respectively (Wilson et al., 1986). A Hind III restriction fragment length polymorphism (RFLP) in the CR1 gene correlates with this expression level. This RFLP is due to a single base change in the intron separating the two exons that encode the second SCR in LHR-D, defining this regulator as an enhancer element that occurs at least 70 kb away from the promoter region of CR1 (A allotype) (Wong et al., 1988). Homozygotes for the L allele generally have fewer than 200 CR1 copies per cell; homozygotes for the H allele have 3-4 times more (Wilson et al., 1986), while heterozygotes have intermediate expression. Influence of this polymorphism on the levels of leucocyte cell surface CR1, however, is not visible, most likely due to high turnover of leucocyte membrane proteins.

**Leucocyte CR1 (L-CR1)**

‘Resting neutrophils’ and monocytes have about 5000 CR1 per cell, although following activation of neutrophils, levels of CR1 are rapidly increased (within a few minutes) by as much as ten fold (Fearon and Caroll, 2000). The enhanced expression occurs in the absence of degranulation and in the presence of protein synthesis inhibitors, implying a non-granule intracellular pool, and is triggered by nanomolar quantities of inflammatory mediators. Immuno-electron microscopy has localized the intracellular CR1 pool to small vesicles (Berger et al., 1996; Kumar et al., 2005). Interestingly, neutrophils release large amounts of soluble CR1, particularly when stimulated with various agents such as FMLP and TNF-α.

B cells express very high levels of CR1 between 20,000 and 40,000 CR1 per cell (Fearon, 1980). These high levels might be necessary to capture immune complexes on B cell surface. CR1 cofactor activity then triggers factor I mediated cleavage to generate C3dg-coated antigen; which in turn binds CR2 and cross links the CD19/CR2 signaling complex with sIgM resulting in B cell proliferation and antibody production.

**Soluble CR1 (sCR1)** with a molecular weight comparable to erythrocyte CR1 has been described in the plasma. sCR1 is also found in the synovial fluid of patients with inflammatory joint disease, where high levels of CR1 in the joint are associated with severe disease. Soluble CR1 is secreted from erythrocytes and leucocytes as a result of the proteolytic cleavage that occurs within the C terminus of the transmembrane region of CR1 in the late secretory vesicles or at the plasma membrane.
**Biological functions of CR1**

**Regulation of complement cascade:** CR1 is involved in the regulation of both the CP and AP, and encompasses the functions of both DAF and MCP by acting as a DAF and a cofactor for factor I-mediated cleavage of C3b and C4b. CR1 binds C3b and C4b, and, with a lower affinity, iC3b and C3dg (Fearon, 1980; Ross et al., 1982). Ligand binding by CR1 leads to complement regulation by accelerating convertase decay. Through binding to C4b, CR1 accelerates the decay of the classical C3 convertase (C4b2a) by displacing C2a. In a similar manner, through the binding of C3b, CR1 accelerates the decay of the alternative pathway C3 convertase (C3bBb) by displacing Bb. The binding also decays the classical pathway C5 convertase (C4b2a3b) and the alternative C5 convertase (C3bBb3b) (Iida and Nussenzweig, 1981). CR1 acts mainly on extrinsic convertases on nearby cells and on ICs whereas MCP and DAF act mainly on C3/C5 convertases on the same cell.

CR1 also contributes to complement regulation by providing co-factor activity for factor I, a serine esterase that cleaves C3b and C4b to hemolytically inactive forms. Specifically, CR1 promotes the cleavage of C3b, by factor I, to C3bi, and further promotes the cleavage of C3bi to C3dg and C3c (Ross et al., 1982; Medof et al., 1984). The CR1 co-factor activity for the generation of C3dg is based on the ability of CR1 to bind C3bi and represents a function not exhibited by any other co-factor under physiological conditions. CR1 also provides co-factor activity for the factor I-mediated cleavage of C4b to C4c and C4d (Iida and Nussenzweig, 1981; Medof et al., 1987). The regulatory function of CR1 on complement cascade is predicted (Khera and Das, 2009) in figure 7.

**Immune complex clearance:** E-CR1 serves as an immune adherence receptor for C3b/C4b-opsonized immune complexes, which, following adherence, are transported to liver and spleen where they are transferred to and processed by fixed macrophages. CR1 on erythrocytes act as a vehicle for clearance of ICs. The ICs altered this way become less pathogenic (Hebert, 1991). The liver is the main site for removal of C3b bearing ICs (Cosio et al., 1990). Kupffer cells trap immune complexes after cleavage of C3b into iC3bor C3dg. CR1 does not have affinity for iC3b. However, CR3 and CR4 present in high density on kupffer cells, bind iC3b. ICs that contain C3dg or C3d may also be trapped by B cells, which express CR2 receptor (Reynes et al., 1985). In addition, follicular dendritic cells can trap complexes bearing iC3b and C3dg.
Figure 7: Regulation of the complement cascade and immune complex clearance by CR1 (A) erythrocyte CR1 involved in complement pathway regulation and immune complex clearance (B) polymorphonuclear (PMN) cell CR1 and monocytes CR1 involved in phagocytosis (C) B-cell CR1 involved in B-cell regulation.


**Phagocytosis:** CR1 expressed on the surface of phagocytic cells may bind soluble polymeric C3b, which is covalently fixed to immune complexes or particles and enhances their phagocytosis (Fearon, 2000). The CR1 and Fc gamma receptors cooperate for phagocytosis of targets that have been coated with suboptimal amounts of IgG. The cross linking of these receptors elicits a number of secondary responses in phagocytic cells. These include neo synthesis and release of arachidonic acid metabolites, stimulation of oxidative burst, release of toxic oxygen derivatives and lysosomal enzymes.

**Role in mediating inhibitory signals to B cells:** Fingeroth et al., 1989 reported that co-cross linking of sIg and CR1 on resting splenic cells results in inhibition of the anti-IgM induced proliferation. Jozsi et al., 2002 also showed that clustering of CR1 via its natural ligand on anti-μ activated human B cells results in generation of inhibitory signals. The mechanism proposed for this inhibition was that, inhibitory molecules (such as FcγRIIb)
co-cluster with CR1 upon engagement of the latter by its activation threshold of B cells. This mechanism may ensure additional level of regulation, which depending on the composition of ICs and the degradation stage of C3 might reduce non-specific B cell activation.

**Regulation of the immune responses:** The stimulation of human monocytes with C3b *in vitro* induces the intracellular production and extra cellular release of IL-1 in serum free conditions. It enhances the differentiation of B cells, but does not have any effect on memory responses.

**Disease association with CR1:**

Several studies (Miyakawa et al., 1981; Thomsen et al., 1987; Walport et al., 1994) including those carried out in our lab (Arora et al., 2000a, 2000b 2001; Katyal et al., 2004; Sivasankar et al., 2004) had documented lower than normal levels of E-CR1 in the patients with glomerulonephritis (GN), Systemic Lupus Erythematosus (SLE) and Rheumatoid Arthritis (RA). Deficiency of CR1 in these diseases is speculated as a major cause for IC non clearance and exaggerated C-activation in these diseases. A prognostic significance for urinary Cr1 and E-CR1 is envisaged (Sivasankar et al., 2004). Simultaneously, soluble recombinant CR1 is found effective in the treatment of inflammatory disease including GN and MI (Weisman et al., 1990; Couser, 1999). It has been since under active investigation to elucidate mechanisms that may underlay the deficient CR1 expression in these diseases. With this, the need for gaining insight into the factors that regulate the normal expression of CR1 was perceived.

**CR1 in Indian context**

i. Studies carried out by the research group in the department of Biochemistry, AIIMS revealed inverse relationship of the erythrocyte (Arora et al., 2001), leucocytes (Arora et al., 2004) and glomerular podocyte CR1 (Sivasankar et al., 2004) with the disease activity of glomerulonephritis including lupus nephritis (Sivasankar et al., 2004). Deficient ECR1 expression in the patients with rheumatoid arthritis was also documented (Arora et al., 2000b). Failures of the complement regulation and IC clearance consequent to CR1 deficiency thereafter were speculated as key events causing pathological manifestation in this disorder.
ii. (Arora et al., 2000a) demonstrated normalization of the CR1 levels in drug responsive patients with better prognosis from a serial study on the patients with glomerulonephritis. (Sivasankar et al., 2004) also suggested diagnostic and prognostic significance of the erythrocyte CR1.

iii. Recombinant soluble CR1 is an effective therapeutic in the treatment of immunoinflammatory disorder and endogenous soluble CR1 is either derived from the proteolytic cleavage of the cell surface CR1 or is secreted from the leucocytes, It was predicted as a disease marker and an index for the status of CR1 expression in health and diseases (Katyal et al., 2001). Simultaneously, the potential of the urinary CR1 as a marker for glomerular damage for partial replacement of repeated biopsies in the end stage renal disease were explored and was found promising as a marker for renal involvement of SLE (Sivasankar et al., 2004).

iv. The experimental evidence to postulate disease - induced associated reduced gene transcription and translation accounted for deficient leukocytic CR1 expression in SLE (Arora et al., 2004) and also documented greater decline in the CR1 transcript in severe form of the disease i.e. Lupus nephritis (Arora et al., 2004). Significant difference in the level of CIC, ANA, ds DNA could be observed in these two groups which correlated significantly with the levels of CR 1 gene transcription which essentially is a novel finding and, needs in depth investigation.

v. Studies have also shown that CR1 expression was increased on monocytes from patients with active RA than controls (Arora et al., 2000b).

CR1 as disease activity marker

CR1 expression gets up regulated with the improvement in the clinical profile of the patients with RA (Arora et al., 2000b) and SLE (Preliminary findings in the lab). However, the levels of CR1 further decline in those patients who either did not respond positively to the medicines or discontinued with the treatment. These patients had the worst prognosis. This immensely suggested usefulness of CR1 as a disease activity marker (Sivasankar et al., 2004; Verma et al., 2005). The surface expression of CR1 on monocytes/macrophages and B cells is strongly reduced in mice infected
with *Plasmodium yoelii*, a rodent malaria model. Monocytes/macrophages from these infected mice present a specific inhibition of complement-mediated internalization of IC caused by the decreased CR1 expression (Fernandez-Arias et al., 2013).

### 2.3.2 Complement Receptor 2 (CR2, CD21)

The complement receptor type II (CD21) belongs to the super gene family of regulators of complement activation. CD21 is the functional receptor for C3d fragments on immune complexes and the Epstein-Barr virus (EBV) envelope protein gp350 (Ahearn and Fearon, 1989). Human CR2 is the receptor for the C3d/C3b in addition to Epstein Bar virus CEBL, CD23 and expressed on all mature B-lymphocytes, follicular dendritic cells and also on T-lymphocytes (Delibrias et al., 1992). CR2 on B-lymphocytes plays an Antigen dependent enhancing role in the immune response by triggering proliferation of preactivated cells and augmenting calcium signals induced in response to cross-linking of SigM (Carter et al., 1988). Functional role of CR2 on T-cells is not clear. Recent evidences emphasize the potential role of receptor on T-cells in the regulation of immune response and infection with lymphocytotropic viruses (Fischer et al., 1991). One of the recent studies suggests that CR1 and CR2 on the surface of B-lymphocytes collaborate to internalize C3b and Ic3B proteins (Grattone et al., 1999).

**Structure of CR2**

Like CR1, CR2 is a member of the RCA family. CR2 is expressed as two alternatively spliced gene products (140 kDa glycoprotein) encoding 15 or 16 SCRs; where the additional SCR in the longer version lies between SCR10 and SCR11 of the shorter CR2 (Iida et al., 1983; Weis et al., 1984). CD21 is expressed on mature B-lymphocytes and B cell lines, but not on early pre and pro B cell and late developmental stages (Tedder et al., 1984). It is also expressed on peripheral blood and thymic T cells (Fischer et al., 1991), T cell lines (Larcher et al., 1990; Delibrias et al., 1992) and a number of other cell types (Carroll and Prodeus, 1998; Fearon, 2000).
Figure 8: Structural features of CD21. The short consensus repeats (SCR) of CD21 are represented as ovals. The exon 11, present as a splice variant in the long form of CD21 is indicated by an arrow. The ligands binding to their respective SCR are shown on the right and the antibodies against respective epitopes are shown on the left.

Source: M. Masilamani, 2002.

Function of CR2

Functionally, CD21 on B cells and follicular dendritic cells (FDC) is implicated in the recognition and binding of immune complexes while the function in T cells and all other cell types is not known (Moir et al., 2000). In T cells, the expression of CD21 is developmentally regulated as double negative thymocytes express membrane bound CD21 (Fischer et al., 1999). Ligation of CD21 results in various signals that are critical for normal B cell responses (Fearon and Carroll, 2000). Crosslinking CD21 with C3d or certain anti CD21 antibodies in the presence of T cell factors leads to B cell proliferation and differentiation (Nemerow et al., 1985; Wilson et al., 1985). Crosslinking CD21 with membrane IgM promotes T cell-independent proliferation (Carter et al., 1988; Fingeroth et al., 1989). On mature B cells, CD21 forms a non-covalent signal transduction complex in the plasma membrane together with the CD81, Leu-13 and the pan-B cell antigen CD19. This complex amplifies the signal transmitted through the B cell receptor by specific antigen and thereby reduces the threshold of antigen necessary to initiate cell proliferation (Dempse et al., 1996; Cherukuri et al., 2001a). The mechanism involved appears to be synergism between the IL-4R and BCR-CD21 signaling pathways in promoting the progression of resting B cells past an early G1 checkpoint (Mongini
Inman, 2001). In addition, CD21 plays a key role in determining B cell survival by limiting apoptosis induced through ligation of membrane IgM (Kozono et al., 1995) and through accumulation of Bcl-2 (Roberts and Snow, 1999).

Alternatively, CD21 participate in the generation of a normal immune response by internalizing and directing C3-bound Ag into the class II processing pathway of B cells (Lanzavecchia et al., 1988; Cherukuri et al., 2001a). CD21 is also shown to have a direct influence on B cell-T cell signal exchange by simultaneous up-regulation of CD80 and CD86 on murine splenic B cells (Kozono et al., 1998). The other functions of CD21 reported in literature though not yet clear are in development and maintenance of B1 cells (Ahearn et al., 1996). In human pro- and pre-B cells the expression of the CD21 gene is silenced by methylation of a CpG island in its promoter. Expression in mature B cells is accompanied by the loss of CpG-methylation (Schwab and Illges, 2001a, 2001b). C3 deposition on B cells may enhance their interaction with CD21 on FDC and vice versa. CD21 in FDC plays a very important role in rescuing antigen-activated B cells from apoptosis (Liu et al., 1989), promotion of somatic hypermutation (Apel and Berek, 1990; Nie et al., 1997) and class switch (Croix et al., 1996; Wu et al., 1996).

CD21 is also found in a soluble form (sCD21) generated by shedding from lymphocytes in culture and in human plasma (Myones and Ross, 1987; Ling et al., 1991; Huemer et al., 1993). Ling et al., 1991 have purified a 72 kDa form of sCD21 from lymphoblastoid cell lines by affinity chromatography on sepharose-coupled BU34, BU33 and BU36 mAbs followed by DEAE ion exchange chromatography. Later, by metabolically labeling the LICR-LON-Hmy cell line with S\(^{35}\), they could isolate several proteins of molecular range from 30-130 kDa. In addition to a range of proteins isolated from tissue culture supernatants, cell-associated CD21 from cell lysates was detected as a 120-140 kDa molecule and was reduced to 115 kDa upon treatment with endoglycosidase (Ling et al., 1991). sCD21 affinity-purified from human serum with THB5 and BU32 mAbs showed a 135 kDa and a 90kDa protein. Moreover, sCD21 circulates as a complex with cleavage fragments of C3 and a trimeric form of soluble CD23 (sCD23). CD21 isolated from human serum showed a smear of 135-190 kDa under non-reducing conditions (Fremeaux-Bacchi et al., 1998b).

**Signaling of CR2**

CR2 possesses only a short cytoplasmic tail and is unlikely to act directly as a signal transducer. However, by virtue of its association with the trimolecular glycoprotein
complex CD19/CD81 (TAPA-1)/Leu 13, CR2 plays a pivotal role in augmenting the B-cell response to opsonized antigen by bringing CD19 into close proximity with the BCR (Guthridge et al., 2001). Colligation of CD19 with BCR reduces the threshold for stimulation via BCR by at least two orders of magnitude. CD19 becomes tyrosine phosphorylated upon ligation with BCR and associates with the protein tyrosine kinases (PTK), Lyn and Fyn, PI3 kinase and the Rac guanine nucleotide exchange factor Vav, which is responsible for activating the MAPK cascade. The enhancement of BCR signal transduction by CD19 is thought to involve at least two elements: (1) phosphorylation, by CD19-bound Lyn, of potential substrates in the BCR complex and (2) Ca2+ mobilization by a PI3 kinase dependent mechanism (Masilamani et al., 2004), distinct from the phospholipase Cγ-mediated mobilization initiated by BCR.

**Disease association of CR2:**

CD21 has been associated to a number of diseases, especially to EBV-related ailments, as CD21 serves as its receptor, and to autoimmune diseases. sCD21 levels are often altered in pathologic conditions including various lymphoproliferative leukemias, such as B-CLL (B cell-type chronic lymphocytic leukemia) (Lowe et al., 1989), acute EBV-infection and other virus-associated diseases (Huemer et al., 1993; Larcher et al., 1995), and autoimmune diseases (Masilamani et al., 2004).

Significantly large expansion of CR2 bearing T-cells (80%) of the total CD3 in a patient with lupus nephritis (Grattone et al., 1999) has been documented decline in CR2 had been reported during the development of autoimmunity in a mouse model but a quantitatively lower level of CR2 gene transcript in the synovial fluid B-lymphocytes from patients with RA had also been documented (Illeges et al., 2000).

B-cell expression of human CR2 and CR1 had been studied in number of human autoimmune diseases (Wilson et al., 1986); one striking finding is that patients with SLE reproducibly demonstrate abnormal expression. B-cells CR2 and CR1 are below 50% of the normal (Wilson et al., 1986; Marquart et al., 1995). It is however, not known whether the disease in B-cell receptor precedes the development on parallels clinical disease activity. EBV is the best studied member of the herpes virus family and is involved in the pathogenesis of several human malignancies, as endemic Burkitt’s lymphoma, nasopharyngeal carcinoma, Hodgkin’s lymphoma, polyclonal lymphomas in immuno-compromised individuals and many more (Khanna et al., 1995; Young et al., 2007). sCD21 has been described as a marker of B-cell activation in humans and
elevated sCD21 levels were found in patients with EBV-associated malignancies. Furthermore, sCD21 can inhibit EBV-binding and infection of B-cells (Nemerow et al., 1990; Huemer et al., 1993). However, as sCD21 is able to bind its ligands in plasma, the increased amounts of sCD21 caused by EBV-infection may contribute to the immunoregulatory dysfunctions observed in EBV-associated diseases (Larcher et al., 1995). Also, CD21 and its soluble form (sCD21) have been described to bind to the low-affinity IgE-receptor CD23 and to sCD23, thereby modulating IgE responses and monocyte activation and differentiation. Therefore, an imbalance of sCD21 and sCD23 may contribute to the development of allergic reactions (Fremeaux-Bacchi et al., 1998; Kwon et al., 2003).

The mechanism by which CD21 might regulate B-cell reactivity to autoantigens has not been clarified yet. It may involve direct effects on B-cell tolerance or indirect effects on T-cell tolerance (Boackle and Holers, 2003). The involvement of CD21/CD35 in the control of self tolerance was shown by Prodeus et al. (Prodeus et al., 1998), where CD21/CD35-deficient mice where crossed in mice expressing transgenic anti-hen-egg lysozyme (HEL) specific antibodies (Hartley et al., 1991). In the presence of both HEL in the blood and anti-HEL transgenic B-cells, B-cells were rendered unresponsive (anergic) in the periphery. CD21-deficient B-cells were able to up-regulate CD86 and the BCR, but still did not respond with antibody secretion (Holersb and Marchbanka, 2009). Hence, CD21 has some role in the induction of anergy. Crossing CD21/CD35-deficient mice with Fas-deficient mice that normally develop autoimmunity of a lupus-like type, renders the offspring with higher anti-DNA autoantibody titers, and thus enhanced autoimmunity (Prodeus et al., 1998). Furthermore, CD21-expression has been linked to enhanced susceptibility for systemic lupus erythematosus (SLE) (Boackle, 2005). As BAFF independently regulates CD21 and CD23 expression (Gorelik et al., 2004), and BAFF overexpression leads to autoantibody production, seen in SLE, primary Sjögren’s syndrome (pSS) and rheumatoid arthritis (RA), some close link between over-activation of B-cells, CD21-expression and the development of autoimmunity may be drawn.

Moreover, treatment of SLE mice with a BAFF protein antagonist ameliorates disease progression and enhances survival (Pers et al., 2005). In patients with SLE, an autoimmune disease characterized by antibodies specific for nuclear antigens such as dsDNA, sCD21 levels are reduced (Masilamani et al., 2004). Low sCD21 levels are also found in the Sjögren’s syndrome, a disease characterized by autoantibodies directed against salivary and lacrimal glands. In juvenile arthritis, though, sCD21 levels were not altered (Masilamani et al., 2004).
These data together with data from patients with autoimmune rheumatoid arthritis having reduced expression levels of CD21 insynovial B- and T-cells (Illges et al., 2000) and significantly lower sCD21 plasma levels (Masilamani et al., 2004), indicate a role for CD21 in autoimmunity. Interestingly, in SLE, pSS and RA, sCD21 levels have been described to be up-regulated (Pers et al., 2005).

**CR2 in Indian context**

To the best of our knowledge, not much is worked on this receptor in India.

**CR2 as disease activity marker**

An inverse relationship between SLE disease activity index (SLEDAI) and the expression of complement receptor 2 (CR2) on SLE; B cells suggest that CR2 on B-cells may emerge as an additional laboratory tool in the assessment of SLE activity (Marquart et al., 1995).

**2.3.3 Relationship between complement receptor 1(CR1, CD35) and complement receptor 2(CR2, CD21)**

Both CR1 and CR2 are expressed by mature B cells (Wilson et al., 1986). The mechanism of complement regulation of the B cells response appears to involve the interaction of the complement activation products (C3b and C3d) and CR1, CR2 on the cell surfaces, increasingly the retention of antigen in lymphoid tissue and enhancing the B cell response. Complement receptors CR1 and CR2, which recognize activated products of C3 and C4, are predominantly expressed on B cells in humans and follicular dendritic cells (FDCs) in the mouse (Roozendaal and Carroll, 2007). Under many conditions, CR1 and CR2 levels on B-lymphocytes as well as CR1 on leucocytes and erythrocytes are reduced (Ross et al., 1985; Benedetto et al., 1992). In fact soluble CR2 is found in the circulation in normal individuals (Huemer et al., 1993; Fremeaux-Bacchi et al., 1996, 1999) and at increased levels in certain diseases (Huemer et al, 1993; Fremeaux-Bacchi et al., 1996). CR2 can be shed and or reduced in copy number on B or T cells and one or more undefined proteases may cut CR2 (Fremeaux-Bacchi et al., 1996, 1999). Thus studies about complement receptor 1 and 2 are needed. The experimental evidence and functional attributes of CR1 and CR2 suggest their immense role in the immune-imbalance in autoimmune disorders and significance as putative disease markers.

The expression of the complement receptor is suggested to play an important role in the pathophysiology of autoimmune disorder. The expression of these receptors may be different in different blood cells in health and disease. Few studies have been carried out
on complement receptor 1; however studies on complement receptor 2 are inadequate. The present study therefore aims at filling in the existing lacunae. The present proposal, the first of its kind would provide baseline data on the expression levels of CR1 and CR2 on the neutrophils, monocytes and lymphocytes in the healthy individuals and its relationship with the pathophysiology and clinical disease activity of Rheumatoid Arthritis. The study thus would facilitate the evaluation of CR1 and CR2 as disease activity markers for Rheumatoid Arthritis.
3. AIM AND OBJECTIVES

Aim

Expression of leucocyte CR1 and CR2 (complement receptor 1 and 2) and its significance in the pathophysiology and clinical disease activity of rheumatoid arthritis.

Objectives

The experimental objectives were to:

a) Study and compare the expression profile of the complement receptors CR1 and CR2 in three different blood cells i.e. lymphocytes, monocytes and neutrophils at cell surface and transcript level in controls and patients suffering from Rheumatoid Arthritis.

b) Study the levels of C3, C3d and CIC in stored plasma and serum samples; relate that with the cell surface and transcript level of CR1 and CR2.

c) Evaluate the correlation among the above parameters and its relationship with the clinical disease activity.
4. METHODOLOGY

4.1 Study Subjects

One hundred patients with the diagnosis of RA and equal number of healthy volunteers (controls) were enrolled for the study.

a) **Controls:** Healthy volunteers, with no history of autoimmune disorders, major infection and other inflammatory diseases were recruited as normals. Among 100 control subjects, 86 were female and 14 were male (age 18 - 41 yrs).

b) **Patients:** The patients were selected from the OPD of Medicine Department, All India Institute of Medical Sciences, New Delhi. The group consisted of 94 females and 6 males (18 - 52yrs). Of these one hundred patients, 17 patients were enrolled for a longitudinal follow up study. Blood from these patients were collected on the day 0 (1st day of diagnosis, treatment naïve) and at the end of the 3rd month and 6th month of treatment. The patients were categorized as follows:

**Group - 1:**

The patients who are coming for the first time to OPD without any medication (n=100).

**Group - 2:**

17 patients were enrolled for longitudinal follow up studies.

**Inclusion Criteria for Controls**

a) Healthy individuals with no known disease.

b) Healthy family members and friends of the patients were included.

c) Age: 18 - 60 years.

**Inclusion Criteria for RA patients**

a) Patients who fulfill the American College of Rheumatology (ACR) revised criteria were included in the study.

b) Age: 18 - 60 years.
Exclusion Criteria for RA patients

a) Pregnant women and patients who were unwilling to participate in the study.

b) Individuals with any acute or chronic infection in the recent past were excluded.

c) Patients having overlap of RA with other systemic autoimmune disease were excluded.

d) Subjects with drug abuse were also excluded.

Clinical details of the patients were obtained from hospital records and DAS28 score was calculated accordingly. Out of 100 RA patients, DAS28 score was available only for 57 patients. The patients were matched as closely as possible for age, sex, lifestyle and geographical lineage with controls excepting that they were suffering from RA. Diagnosis and recruitment of patients for this study was done by the clinicians. The details of the patients remained encoded till study progressed to a decisive end.

Diagnosis was done according to the criteria put forward by ACR for the classification of RA (Arnett et al., 1988). The patients fulfilled at least four of the criteria either simultaneously or over a period of time. Laboratory investigations like serum and plasma levels of C3, anti-CCP, CRP, ESR, TJC (tender joint count), SJC (swollen joint count) and VAS were done for all the patients in the clinical investigation laboratory. DAS28 score was used to calculate the disease activity. Written consent was taken from each individual before taking blood. The study was approved by the Ethics committee of the All India Institute of Medical Sciences.

4.2 Sample Collections

Venous blood (5 - 7 ml) with or without anticoagulant (EDTA) was drawn from the controls and RA patients. The fluid fraction of blood (Plasma and serum) was separated from the cellular fraction by centrifugation at ~500xg at 4°C, aliquotted and stored at -70°C until further used for the estimation of C3, C3d and CIC levels. The packed cellular fraction was used for the isolation of PBMCs and neutrophils to determine the levels of CR1 and CR2.
4.3 Preparation of the Samples

4.3.1 Isolation of PBMCs and Neutrophils

Freshly heparinized venous blood (7 ml) was obtained from healthy volunteers and patients. The PBMCs and neutrophils were isolated from the whole blood by Ficoll-histopaque density centrifugation according to the method described by Arora et al., 2004. The blood was first diluted in 1:2 ratios with PBS and then carefully layered on the top of the Ficoll-histopaque (density 1.077g/ml) in the ratio 3:1 in a 50 ml falcon tube, which was then centrifuged for 30 minutes at 600Xg at 4°C in a swinging bucket rotor. PBMCs were collected from the PBS/Ficoll interphase with a transfer pipette.

From the 50 ml falcon tube the remaining diluted plasma and ficoll were carefully aspirated down to the RBC pellet, which also contains neutrophils. These cells were lysed by adding Red Cell Lysis buffer and centrifuged at 600Xg for 15 minutes. The obtained cells were neutrophils, washed with PBS. PBMCs and neutrophils were counted before every experiment and tested for viability under light microscope by trypan blue exclusion method on Neubauer hemocytometer counting grid. The isolated PBMCs and neutrophils were then used for CR1 and CR2 estimation by flow cytometry and RNA extraction.

4.3.2 Quantification of CR1 and CR2 on leucocyte cell surface by Flow cytometry

Whole blood leucocytes were analyzed for the cell surface expression of CR1 and CR2 on different cell types by Flow cytometry.

Calibration of the instrument

Flow cytometry was performed on a fluorescence activated cell analyzer (BD FACs Canto). The alignment and calibration of the instrument was checked by using fluorescent calibration beads (BD biosciences) to ensure proper focusing and alignment of all lenses in the path for both the exciting light source and signal (light scatter, fluorescence, etc.) detectors. The flow cytometer was adjusted with the florescence microbeads to yield a constant mean florescence.

Cell preparation

Fresh venous blood was taken and PBMCs and neutrophils were isolated as described earlier. Cells were counted with hemocytometer and the viability was checked by
Methodology

trypan blue staining. 1.1× 10⁶ cells were taken in a micro centrifuge tube and washed twice with PBS (pH 7.4). The cell suspension was centrifuged at 900 x g for 10 min; supernatant was discarded. After washing, cells were suspended in PBS (pH 7.4) containing 2% BSA (PBS-BSA) for blocking.

Antibody staining

The PBMC and neutrophils were incubated for 90 minutes at 4°C in separate tubes with monoclonal primary antibodies (Serotec) specific for CR1 and CR2 and also the corresponding antibodies of the same isotype (Serotec). Isotype –matched mouse antibodies were used to calculate the non –specific staining and acted as isotype control. The cells were then washed with 2%PBS-BSA followed by incubation with FITC conjugated secondary antibodies for 30 minutes in dark. Stained cells were washed twice with 2%PBS-BSA followed by PBS (PH 7.4) and fixed with 2% paraformaldehyde in PBS (PH 7.4) till further analysis. The labeled cells were analyzed within a short time period to avoid significant change in test results.

Acquisition and analysis

Stained cells were acquired in flowcytometer using FACS Analyser (BD FACS Canto). Results were analyzed using BD FACS Diva software. Neutrophils, monocytes and lymphocytes have distinct light-scattering properties owing to their difference in size and granularity and hence they appeared as distinct clusters in a side scatter vs forward scatter plot (Robinson, 2004). Each cell type was gated for measurement of fluorescence intensity independent of the other cell types on the basis of forward and side scatter. Besides detecting the light scattering from individual cell, a fluorescence signal, adapted from FITC emission (Its excitation by 488-nm light leads to a fluorescence emission maximum around 520 nm), was detected as the cells passed the laser beam. The instrument gives the percentage, actual number of cell and mean fluorescence intensity (MFI) of the cell population within each of the three clusters. The results were expressed as MFI and were calculated by subtracting the mean florescence of cells stained with the isotype control antibody from the florescence of the cells stained with the CR1 and CR2 specific antibody. 10,000 cells were analyzed from each sample. Each set was performed in duplicates.
**4.3.3 Isolation of RNA from various cell populations**

**RNA Isolation**

Precautions taken for RNA isolation and processing. It was ensured that all plastic wares and reagents used for RNA isolation and all its downstream processing of RNA were RNase free. All plastic wares including micropipette, pipette tips, tip boxes, centrifuge tubes and microfuge tubes were treated with 0.1% DEPC (Diethyl pyrocarbonate) for overnight at room temperature followed by removal of DEPC through autoclaving. RNase free MQ water and PBS (pH 7.4) were prepared in the same fashion by treatment with 0.1% DEPC and autoclaving. Disposable plastic gloves were used during handling of materials to prevent RNase contamination.

RNA isolation from PBMCs and neutrophils: RNA was isolated using Tri Reagent (Sigma-Aldrich, USA) which is based on the single-step method as reported by Chomczynski, 1993 for total RNA isolation. Tri Reagent is the mixture of guanidine thiocyanate and phenol in a mono-phase solution effectively dissolves DNA, RNA, and protein on homogenization or lysis of the sample. 1 ml of Tri Reagent was used to homogenize a pellet containing $1 \times 10^7$ cells (PBMCs / neutrophils). 0.2 ml of chloroform was added to the homogenate with vigorous vortexing followed by centrifugation at 12,000xg and 4°C for 15 minutes. This lead to the separation of the mixture into 3 phases: a red organic phase (containing protein), an interphase (containing DNA), and a colorless upper aqueous phase (containing RNA). RNA was isolated from the aqueous phase of each sample according to the manufacturer’s instruction. The extracted RNA was quantified and integrity evaluated.

**Quantification and analysis of RNA:** Concentrations of RNA in solution were measured by spectrophotometry or nanodrop. Samples were diluted in 1 ml autoclaved DDW and the optical densities (OD) were measured at 260 nm and at 280 nm in spectrophotometer. The samples having the OD ratio at 260 and 280 nm in the range of 1.9 to 2.2 were taken for reverse transcription. 1 OD at 260 nm was considered to represent 40 µg/ml of RNA. Concentrations of the RNA samples were calculated from the OD by using corresponding dilution factors. Samples were also measured for RNA concentration in a nanodrop (ND-1000), which uses 1-2 µl samples to measure the RNA concentration spectrophotometrically. The integrity and size distribution of the RNA samples was checked by running the samples on the formaldehyde gel.
RNA gel electrophoresis: RNA electrophoresis was performed under denaturing conditions in 2.2 M formaldehyde according to Arora et al., 2004 using the MOPS buffer system. RNA under these conditions is fully denatured and migrates according to the log_{10} of its molecular weight. The overall quality and yield of the RNA preparation was assessed by electrophoresis on a denaturing agarose gel.

Estimation of CR1 and CR2 transcripts

RTPCR was performed according to the method of Arora et al., 2004 with minor modifications.

cDNA synthesis (Reverse Transcription)

First strand cDNA was synthesized by using oligo-dT and Expand RT reverse-transcriptase enzyme kit (Rosche Diagnostics). The sample mixture was prepared as given in the following table (Table. 11).

Table 11: Sample Mixture for Reverse Transcription

<table>
<thead>
<tr>
<th>Amount</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-2 ug</td>
<td>Total RNA</td>
</tr>
<tr>
<td>q.s. to 10 ul</td>
<td>Nuclease free water</td>
</tr>
</tbody>
</table>

The sample mix was incubated at 65°C for 10 minutes to break RNA secondary structures and immediately chilled on ice-water and kept for few minutes. The tubes were pulsed briefly and kept back on ice. The remaining RT components were prepared as given in the following table (Table. 12)

Table 12: Components of Reverse Transcription

<table>
<thead>
<tr>
<th>Amount</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 ul</td>
<td>5x RT buffer</td>
</tr>
<tr>
<td>1 ul</td>
<td>10 mM dNTP mix</td>
</tr>
<tr>
<td>1 ul</td>
<td>Oligo dT</td>
</tr>
<tr>
<td>1 ul</td>
<td>5 mM DTT</td>
</tr>
<tr>
<td>1 ul</td>
<td>RNase inhibitor</td>
</tr>
<tr>
<td>1 ul</td>
<td>Reverse Transcriptase (AMV-RT)</td>
</tr>
</tbody>
</table>

8 µl of the remaining RT components; were added to the heat-treated sample mixture making the final volume to 20µl. The reverse transcription was carried out at 42°C for
60 minutes and the reaction was stopped by incubating the RT-mixture at 94°C for 5 minutes (which inactivates the Reverse Transcriptase) in PCR machine (MJ Research Thermal Cycler (Model PTC –100™). The RT-mixture was stored at -20°C till used for downstream PCR amplifications. A negative control reverse transcription reaction was also performed without adding reverse transcriptase in the RT-mixture.

**Polymerase Chain Reaction (PCR)**

RT-PCR was performed with specific primer-pairs to target CR1, CR2 and β-actin transcripts using the cDNA as template. β-actin was used as an internal control. The sequence of the primers, its annealing temperature and product length is described in the table below. PCR with β-actin primers was done for all cDNA samples to check for any DNA contamination. These intron-spanning β-actin primers amplify a product of 172 bp from cDNA and 1.2 kb from genomic DNA. Recombinant *Taq* DNA polymerase was used for all PCR reactions. The reaction was carried out in 25 µl final reaction volume using 1 µl of cDNA, 20 picomole of each oligonucleotide with 200 µM dNTPs and 4 U of Taq DNA Polymerase. PCR was performed in 25 µl of reaction volume as given in the following table (Table. 13).

**Table 13: Reaction Mixture for PCR**

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDNA or DNA template</td>
<td>2 µl (of RT-mix) or ~100ng (of Gen. DNA)</td>
</tr>
<tr>
<td>Primers</td>
<td></td>
</tr>
<tr>
<td>Forward (25pmoles/ µl)</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>Reverse (25pmoles/ µl)</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>10X PCR buffer with (NH₄)₂SO₄</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>[750 mM Tris-HCL (pH 8.8 at 25°C)</td>
<td></td>
</tr>
<tr>
<td>200 mM (NH₄)₂SO₄,0.1% Tween 20]</td>
<td></td>
</tr>
<tr>
<td>MgCl₂ (25mM)</td>
<td>1.5 µl</td>
</tr>
<tr>
<td>dNTP (10mM each)</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>Taq DNA polymerase (5U/µl)</td>
<td>0.33 µl</td>
</tr>
<tr>
<td>Autoclaved DDW q.s to</td>
<td>25 µl</td>
</tr>
</tbody>
</table>

PCR reaction conditions were given in the following table (Table 14).
Table 14: Reaction Programme for PCR

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>94°C</td>
<td>3 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>1 min</td>
</tr>
<tr>
<td>Annealing</td>
<td>58°C</td>
<td>1 min</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>1 min</td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C</td>
<td>10 min</td>
</tr>
</tbody>
</table>

An initial step of heat denaturation (94°C for 3 minutes) was given in every PCR reaction. The PCR conditions were: Initial denaturation at 94°C for 3 minutes and then amplification was carried out for 35 cycles. Each cycle consisted of denaturation for 1 minute at 94°C, annealing for 1 minute at different annealing temperatures specific for a particular gene for different primers and extension for 2 minutes at 72°C. The procedure was ended by a 10 minute final extension step at 72°C. The amplification of each gene was done in the linear manner. The following table (Table 15) depicts the sequence of primer pairs used for RT-PCR along with their annealing temperature and product size.

Table 15: Description of Primer Sequence with Annealing Temperature and Product Size

<table>
<thead>
<tr>
<th>Primers</th>
<th>Description (5’-3’)</th>
<th>Annealing temp (°C)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-actin F</td>
<td>aga aaa tct ggc acc aca cc</td>
<td>53.8</td>
<td>172</td>
</tr>
<tr>
<td>B-actin R</td>
<td>tag cac agc cag gat ag cca</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CR1 F</td>
<td>ccc ttt gga aaa gca gta aa</td>
<td>53.8</td>
<td>193</td>
</tr>
<tr>
<td>CR1 R</td>
<td>tca act tgg caa aca gaa aa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CR2 F</td>
<td>gcc gac acg act acc aac c</td>
<td>59.5</td>
<td>150</td>
</tr>
<tr>
<td>CR2 R</td>
<td>agc aag taa cca gat tca cag c</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Agarose gel electrophoresis of target products

RT-PCR products were resolved on 1.5% agarose gel containing ethidium bromide (0.5 μg/ml) in 1×Tris-acetate-EDTA (TAE) buffer and separated by electrophoresis at 60 volts for 1-2 hrs until desired resolution was achieved. DNA ladder (100bp) was run on each gel. The bands were then visualized under UV transilluminator and documented using Chemi-Imager software in gel documentation system (Biorad, USA). The amplified RT-PCR product for each set of primers was confirmed by the appearance of a dominant
single band of the appropriate size (172 bp for β-actin, 193 bp for CR1 and 150 bp for CR2) on ethidium bromide stained agarose gels. Semi quantitative analysis was conducted using a computerized densitometry imager to obtain integrated density values of the CR1, CR2 and β-actin bands (Biorad; Quantity one software). CR1 and CR2 expression were calculated based on band intensity of RT-PCR products for CR1 and CR2 to the simultaneously amplified β-actin on agarose gel. The level of expression was calculated by taking CR1 or CR2/β-actin ratio multiplied by 100.

4.4 Levels of Circulating Immune Complexes (CIC)

CIC levels in plasma samples of RA patients and controls were estimated by the method described by Sai Baba et al., 1990. In this method, CIC is precipitated by incubating 50µl serum with equal volume of 5% PEG-6000 (polyethylene glycol, final concentration 2.5%) at 4°C overnight and the concentration measured in a spectrophotometer using aggregated human gamma globulin as standard.

4.4.1 Preparation of aggregated human gamma (γ) globulin

Human γ globulin was diluted to 10mg/ml in PBS (pH 7.4). The solution was heated at 63°C for 30 minutes and immediately cooled on ice for 15 minutes. Large aggregates were removed by centrifugation at 600xg for 15 minutes at 4°C. Supernatant was collected and concentration of aggregated globulin determined in the supernatant by using BSA as standard. A standard curve for BSA was obtained by taking OD for different concentrations at 280 nm.

4.4.2 Preparation of standard curve

Heat aggregated globulin supernatant was mixed with equal volume of 5% Polyethylene glycol (PEG) to get a final concentration of 2.5% and tubes were incubated overnight at 4°C. The supernatant was discarded and precipitate washed twice with 2.5% PEG. The residues were dissolved in 1ml of PBS (pH 7.4) for 30 minutes at 37°C. The dissolved precipitate was serially diluted in PBS (pH 7.4) to make a concentration range of 50 to 1000 µg/ml. OD of these dilutions was taken at 280nm to prepare the standard curve for CIC estimation.

4.4.3 Estimation of CIC in controls and patients

The CIC levels were determined from a standard curve plotted using aggregated human gamma globulins as standard. Plasma samples were diluted such that OD readings
would fall within range of standard curve. CIC levels were then estimated in the samples and expressed in terms of μg/ml.

4.5 Estimation of C3

C3 level was estimated by using Mininephplus™ kit (The binding site, Grassobio, Italy) according to the manufacturer’s instructions by nephlometry in the Department of Medicine, AIIMS.

4.6 Estimation of C3d

The complement C3 fragment, C3d (35 kDa), a cleaved product of C3b, shares common epitopes with intact C3 and C3b. C3d levels were estimated using BMASAY ELISA Kit (Biomedical Assay Co. Ltd. Beijing, China) according to the manufacturer’s instructions.

4.7 Statistical Analysis

The results are presented as means ± standard deviation (SD) and in percentages. The differences in averages between the study groups were examined by the Mann-Whitney test and independent sample t-test. The comparison between the two data in follow-up study was done by paired t-test. Correlation coefficient between the test parameters was assessed by applying Spearman's rank correlation test and the significance level was measured by two-tailed paired student’s t-test. In all cases, P <0.05 was considered significant. Data analysis was performed using SPSS software version 14 and Graph pad PRISM version 5.
5. RESULTS

5.1 Standardization of Methods

The methods used in the study to estimate the levels of various clinical parameters and to determine the expression and modulations of CR1 and CR2 were standardized before the actual commencement of the experiments. The standardizations are described in brief.

5.1.1 Semi-quantitative RT-PCR

Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) was performed to determine the levels of CR1 and CR2 transcripts.

5.1.2 Analysis of total RNA: The integrity and quality of the RNA isolated from PBMCs and neutrophils were checked by running the RNA samples on formaldehyde agarose gel. 28S (~4 kb) and 18S (~2 kb) bands of ribosomal RNA (rRNA) were observed. A two-fold intense 28S rRNA band when compared to 18S rRNA indicated intact RNA sample.

![Agarose gel electrophoresis of total RNA. 5 μg of total RNA was run on 1.5% formaldehyde agarose gel. Lane 1: degraded RNA sample, Lane 2: intact RNA sample having 18S and 28S ribosomal RNA bands. M: RNA molecular weight marker.](image-url)
5.1.3 **Optimum conditions for PCR reactions:** PCR amplification of the CR1, CR2 and β-actin genes were carried out with the specific primers. Standardization was done in order to determine the optimum conditions for specific amplification of CR1, CR2 and β-actin genes. RT-PCR was standardized for annealing temperature, cDNA concentration and primer concentration.

5.1.4 **RT-PCR for CR1:** PCR amplification of the CR1 gene with specific primer pair yields a 193 bp product and β-actin with 172 bp products. Annealing temperature, primer concentration, cDNA concentration and PCR cycles for CR1 were previously standardized in our lab (Arora et al., 2004). However, needful modifications were done.

![Image of gel pattern](image_url)

**Figure 5.1.4:** Representative gel pattern of CR1 transcript determined by RT-PCR. The gel depicts the expression of CR1 transcript (lane 1, 2 and 3) and β-actin (lane 4, 5 and 6) in triplicates. Levels of CR1 transcript were expressed as % levels of CR1/β-actin determined by densitometry of the gel bands obtained after RT-PCR. M: DNA Marker (50bp – 1000bp).

- 53.8°C annealing temperature, 12.5 pico moles of each primer, 35 PCR cycles for CR1, 35 PCR cycles for β-actin and 1 µl of the cDNA were found to be the optimum for RT-PCR reaction

5.1.5 **RT-PCR for CR2:** PCR amplification of the CR2 gene with specific primer pair yields a 150bp product. Various primer concentrations (3.12, 6.25, 12.5 and 25 pico moles) and annealing temperature (gradient PCR - 57°C, 58°C, 58.7°C, 59.5°C, 60.3°C, 61.1°C, 62.5°C, 63°C) were used to determine the optimum PCR conditions. cDNA
concentration and PCR cycle were same as that used for RT-PCR for CR1. 1 µl cDNA, 59.5°C annealing temperature and 12.5 picomoles primer concentrations were found to be optimum as they gave specific amplification of the target band with no non-specificity. Hence these conditions were used for further RT-PCR.

**Figure 5.1.5 (A): Standardization of optimum conditions for CR2 RT-PCR (Annealing temperature).** The lanes show CR2 amplification at annealing temperatures of 57°C, 58°C, 58.7°C, 59.5°C, 60.3°C, 61.1°C, 62.5°C and 63°C (lane 1 to lane 8) respectively. M: DNA Marker (100bp-1000bp). 59.5°C annealing temperature were found to be the optimum for CR2 RT-PCR reaction.

**Figure 5.1.5 (B): Standardization of optimum conditions for CR2 RT-PCR (Primer concentration).** Lanes in triplicate show different primer concentrations (25, 12.5, 6.25, 3.12 pico moles) for CR2 RT PCR products. M: DNA Marker (100bp-1000bp). At 12.5 pico moles of each primer was found to be the optimum for CR2 RT-PCR reaction.
Results

Figure 5.1.5 (C): Standardization of optimum conditions for CR2 RT-PCR. The gel depicts the expression of CR2 transcript (lanes 1, 2 and 3), β-actin (lanes 4, 5 and 6) in triplicates. Levels of CR2 transcript were expressed as % levels of CR2/β-actin determined by densitometry of the gel bands obtained after RT-PCR. M: DNA Marker (100bp-1000bp).

5.1.6 Determining CR1 and CR2 in PBMCs and neutrophils by flow cytometry

Flow cytometry was performed to determine the levels of CR1 and CR2 in PBMCs and neutrophils in a flow cytometer (BD FACS Canto).

Gating of the cell population was done on the basis of forward and side scatter. Based on the light scattering properties, each cell was represented by a point in the rectangular co-ordinate system (dot blot). Lymphocytes, monocytes and neutrophils appeared as three distinct clusters in the dot blot. Following Figures depict the histograms showing the MFI for CR1 and CR2 on different cell population. Non-specific staining was ruled out by running a parallel set of cells stained with isotype antibody for each experiment. The control and patient samples show a shift in the peak of cells stained with CR1 and CR2 antibodies with respect to the isotype control in lymphocytes, monocytes and neutrophils. Experiments for each sample were performed in duplicates.

Figure 5.1.6 (A): Dot blot of PBMCs in FACS analysis; Figure 5.1.6 (B): Dot blot of Neutrophils in FACS analysis.
5.1.6 (A) Lymphocytes

![Lymphocytes chart](image.png)

5.1.6 (B) Monocytes

![Monocytes chart](image.png)

5.1.6 (C) Neutrophils

![Neutrophils chart](image.png)

**Figure 5.1.6 (A, B and C):** Representative histograms showing mean fluorescence intensity (MFI) for CR1 on A. lymphocytes B. monocytes and C. neutrophils labeled with specific antibody. Left panels (isotype controls) and right panels (stained samples) show the shift in the peak of cells stained for CR1 with respect to the isotype control in all the cell types.
5.1.6 (D) Lymphocytes

![Histograms showing mean fluorescence intensity (MFI) for CR2 on lymphocytes labeled with specific antibody.](image)

5.1.6 (E) Monocytes

![Histograms showing mean fluorescence intensity (MFI) for CR2 on monocytes labeled with specific antibody.](image)

5.1.6 (F) Neutrophils

![Histograms showing mean fluorescence intensity (MFI) for CR2 on neutrophils labeled with specific antibody.](image)

**Figure 5.1.6 (D, E and F):** Representative histograms showing mean fluorescence intensity (MFI) for CR2 on D. lymphocytes E. monocytes and F. neutrophils labeled with specific antibody. Left panels (isotype controls) and right panels (stained samples) show the shift in the peak of cells stained for CR2 with respect to the isotype control in all the cell types.
5.1.7 Standard curve and validation of assay for CIC

The validation of spectrophotometric assay was done to estimate circulating immune complexes.

**Standard Curve:** The composite standard curve of heat-aggregated γ-globulin (agg-HIgG) for six different spectrophotometric experiments is shown below. The sensitivity of the assay ranges from 50 to 1400 µg/ml. The CIC levels for the patients and control samples were subsequently estimated from the standard curve.

**Sample Dilution Curve and Parallelism with the Standard Curve:** Serial dilutions (ranging from 1:1.25 to 1:40) of the plasma samples of both controls and patients were plotted on the standard curve. The plasma dilution curves of both controls and patients overlapped with the standard curve. The CIC concentration obtained from 1:2.5 dilutions for normal and the patient plasma samples were found in the central part of the plot and hence chosen as optimum for further experiments.

**Stability of the Stored Samples:** No significant variation in the stability of the CIC in plasma from the control and the patient samples was observed upon storage at -70°C till a year.

**Figure 5.1.7 (A):** Composite standard curve for heat aggregated γ-globulins. Six different spectrophotometric experiments were carried out at different times. Each point indicates the mean value of agg-HIgG at a particular concentration. Bars indicate standard deviation (SD) of the mean value. The values for the samples were calculated in reference to the standard curve and expressed in terms of µg/ml.
Figure 5.1.7 (B): Parallelism between agg-HIgG (standard) and normal pooled plasma dilution curve. The black rhombus denotes the values of the standard curve and grey squares denote values for the control plasma samples with the bars showing the standard deviation.

- 1:2.5 dilutions were used for CIC quantitation in controls.

Figure 5.1.7 (C): Parallelism between agg-HIgG (standard) and pooled patient plasma dilution curve. The black rhombus denotes the values of the standard curve and grey squares denote values for the patient plasma samples with the bars showing the standard deviation.

- 1:2.5 plasma dilutions were used for CIC quantitation in patients.
5.2 Characteristics of the Patients

5.2.1 Study Subjects

One hundred patients with the diagnosis of RA and equal number of healthy volunteers (controls) were enrolled for the study.

a) **Controls:** Healthy volunteers, with no history of autoimmune disorders, major infection and, other inflammatory diseases were recruited as controls. Among 100 control subjects, 86 were female and 14 were male (age 18 - 41 yrs).

b) **Patients:** The patients were selected from the OPD of Medicine Department, All India Institute of Medical Sciences, New Delhi. The group consisted of 94 females and 6 males (18 - 52yrs). Of these one hundred patients, 17 patients were enrolled for a longitudinal follow up study. Blood from these patients were collected on the day 0 (1st day of diagnosis, treatment naïve) and at the end of the 3rd month and 6th month of treatment. The patients were categorized as follows:

**Group - 1:** The patients who are coming for the first time to OPD without any medication (n=100).

**Group - 2:** 17 patients were enrolled for longitudinal follow up studies.

Clinical details of the patients were obtained from hospital records and DAS28 score was calculated accordingly. Out of 100 RA patients we calculated, DAS28 score was available only for 57 patients.

5.3 Clinical parameters

Apart from the laboratory investigations done in the hospital, a few additional serological parameters like CIC, C3, C3d and DAS28 score were measured in both controls and RA patients.

5.3.1 Distribution of circulating immune complex (CIC) in controls and 0 months (naïve RA)

The levels of circulating immune complex were measured in all 100 controls and 100 RA patients. The levels of CIC were significantly increased by 52.1% in RA patients as compared to controls (p<0.0001, Mann-Whitney test) (Figure 5.3.1). The CIC levels were
Results

found to be 108.9% higher in patients (mean $1110 \pm 154.3$) than in controls (mean $531.3 \pm 119.7$). In patients and controls, plasma CIC levels ranged from 50-1400 µg/ml.

![Figure 5.3.1: Levels of circulating immune complex.](image)

**Figure 5.3.1: Levels of circulating immune complex.** *The plot shows the absolute individual values of CIC in both controls (n=100) and RA patients (n=100). Each symbol represents one control (indigo triangles) or RA patient (pink stars), and the bar within each group represents the mean value. The P values were derived by Mann-Whitney test.*

### 5.3.2 Distribution of C3 in controls and 0 months (naïve RA)

The level of C3 was estimated in 100 controls and 100 RA patients by nephelometry in serum samples in the Department of Medicine, AIIMS. The mean levels of C3 were found to be $0.708 \pm 0.181$ g/l in controls and $1.373 \pm 0.373$ g/l in RA patients. Hence there was a significant ($p<0.0001$, Mann-Whitney test) (Figure 5.3.2) increase in 93.7% in the level of C3 in RA patients.

![Figure 5.3.2: Levels of C3.](image)

**Figure 5.3.2: Levels of C3.** *The plot shows the absolute individual values of C3 in both controls (n=100) and RA patients (n=100). Each symbol represents one control (indigo triangles) or RA patient (pink stars), and the bar within each group represents the mean percentage value. The P values are derived by Mann-Whitney test.*
5.3.3 Distribution of C3d in controls and 0 months (naïve RA)

The level of C3d was estimated in 32 controls and 33 RA patients by using BMASAY ELISA Kit (Biomedical Assay Co. Ltd. Beijing, China) according to the manufacturer’s instructions. The mean levels of C3d were found to be 35.56 ± 20.68 AU/L in controls and 70.34 ± 16.79 AU/L in RA patients. Hence there was a significant (p<0.0001, Mann-Whitney test) increase in 49.4% in the level of C3d in RA patients.

![Figure 5.3.3: Levels of C3d. The plot shows the absolute individual values of C3d in both controls (n=32) and RA patients (n=33). Each symbol represents one control (indigo triangles) or RA patient (pink stars), and the bar within each group represents the mean percentage value. The P values are derived by Mann-Whitney test.](image)

5.3.4 Levels of DAS 28 score

The DAS28 score were obtained from clinical records of the patients. The DAS28 measures disease activity on a scale from 0 to 10. DAS28 above 5.1 means high disease activity, whereas lower than 2.6 indicates low disease activity. Remission is achieved by a DAS28 lower than 2.6 (comparable to the ARA remission criteria). The DAS28 can be calculated using the following formula (http://www.das-score.nl/):

\[
\text{DAS28} = 0.56 \times \sqrt{\text{tender28}} + 0.28 \times \sqrt{\text{swollen28}} + 0.70 \times \ln(\text{ESR}) + 0.014
\]

5.4 Levels of CR1 transcript in PBMCs and Neutrophils

The level of complement receptor 1 (CR1) was determined in 100 controls and 100 RA patients by semi-quantitative RT-PCR with β-actin as an internal control. The expression of CR1 was calculated in terms of percentage of β-actin transcript. There was a significant decrease in the expression of CR1 in RA patients in both PBMC and neutrophils when compared to controls (p<0.0001, Mann-Whitney test). Mean values for PBMC CR1 in controls was 69.58 ± 19.04 and that of patients was 51.84 ± 13.72
Results

(Figure 5.4A) and in neutrophils CR1 the mean values in controls was $71.23 \pm 20.47$ and that of patients was $52.01 \pm 13.39$ (Figure 5.4B) respectively.

![Figure 5.4 (A and B): Levels of CR1 in PBMCs and Neutrophils determined by semi-quantitative RT-PCR.](image)

The plot shows the values of CR1 expression normalized by β-actin expression in controls (n=100) and RA patients (n=100). Each symbol represents one control (indigo triangles) or patient (pink stars), and the bar within each group represents the mean value. The $P$ value was determined by Mann-Whitney test.
5.4.1 Correlation of CR1 with clinical parameters

The relationship of PBMC and neutrophil CR1 transcript levels with CIC, C3 and C3d were evaluated in both controls and patients.

5.4.1 (i) Correlations of PBMC and Neutrophil CR1 with CIC

In RA patients, CR1 transcript in PBMC correlated significantly with CIC levels in controls \( (p=0.0155, r=-0.2415, \text{spearman rho analysis}) \) (Figure 5.4.1 i A) as well as in patients \( (p=0.023, r=-0.2273, \text{spearman rho analysis}) \) (Figure 5.4.1 i B). However, there was no significant correlation between CR1 transcript in neutrophil with CIC levels in controls \( (p=0.0677, r=0.1835, \text{spearman rho analysis}) \) (Figure 5.4.1 i C) as well as in patients \( (p=0.7303, r=-0.03490, \text{spearman rho analysis}) \) (Figure 5.4.1 i D).

![Figure 5.4.1(i) (A and B): Correlation between CIC levels and CR1 PBMC transcript in controls and RA patients.](image)

Panels A and B represent correlation between CIC levels and CR1 PBMC transcripts in controls \( (n=100) \) and RA patients \( (n=100) \) respectively. The \( P \) and \( r \)-values are indicated in the respective panels.

![Figure 5.4.1(i) (C and D): Correlation between CIC levels and CR1 neutrophil transcript in normal controls and RA patients.](image)

Panels A and B represent correlation between CIC levels and CR1 neutrophil transcripts in controls \( (n=100) \) and RA patients \( (n=100) \) respectively. The \( P \) and \( r \)-values are indicated in the respective panels.
5.4.1 (ii) Correlations of PBMC and Neutrophil CR1 with C3

In RA patients, CR1 transcript in PBMC correlated significantly with C3 levels in controls (p=0.0152, r=-0.2423, spearman rho analysis) (Figure 5.4.1 ii A). However, there was no significant correlation between PBMC CR1 transcript in with C3 levels in patients (p=0.2428, r=0.1179, spearman rho analysis) (Figure 5.4.1 ii B). There is no significant correlation between CR1 transcript in neutrophils in both control (p=0.1519, r=0.1419, spearman rho analysis) and in patients (p=0.7445, r=-0.0329, spearman rho analysis) (Figure 5.4.1 ii C and D).

Figure 5.4.1 (ii) (A and B): Correlation between C3 levels and CR1 PBMC transcript in normal controls and RA patients. Panels A and B represent correlation between C3 levels and CR1 PBMC transcripts in controls (n=100) and RA patients (n=100) respectively. The P and r- values are indicated in the respective panels.

Figure 5.4.1 (ii) (C and D): Correlation between C3 levels and CR1 neutrophil transcript in normal controls and RA patients. Panels C and D represent correlation between C3 levels and CR1 neutrophil transcripts in controls (n=100) and RA patients (n=100) respectively. The P and r- values are indicated in the respective panels.
5.4.1 (iii) Correlations of PBMC and Neutrophil CR1 with C3d

In RA patients, CR1 transcript in PBMC correlated significantly with C3d levels in controls ($p=0.034$, $r=0.374$, spearman rho analysis) and in patients ($p=0.018$, $r=0.409$, spearman rho analysis) (Figure 5.4.1 iii A and B). There is a significant correlation between CR1 transcript in neutrophils in both control ($p=0.004$, $r=0.491$, spearman rho analysis) and in patients ($p=0.027$, $r=0.384$, spearman rho analysis) (Figure 5.4.1 iii C and D).

![Correlation between C3d levels and CR1 PBMC transcript in normal controls and RA patients.](image1)

**Figure 5.4.1 (iii) (A and B): Correlation between C3d levels and CR1 PBMC transcript in normal controls and RA patients.** Panels A and B represents correlation between C3d levels and CR1 PBMC transcripts in controls ($n=32$) and RA patients ($n=33$) respectively. The $P$ and $r$-values are indicated in the respective panels.

![Correlation between C3d levels and CR1 neutrophil transcript in normal controls and RA patients.](image2)

**Figure 5.4.1 (iii) (C and D): Correlation between C3d levels and CR1 neutrophil transcript in normal controls and RA patients.** Panels C and D represent correlation between C3d levels and CR1 neutrophil transcripts in controls ($n=32$) and RA patients ($n=33$) respectively. The $P$ and $r$-values are indicated in the respective panels.
5.5 Level of CR2 transcripts in PBMCs and Neutrophils

There was a significant decrease in the expression of CR2 in RA patients when compared to controls (p<0.0001, Mann-Whitney test) in both PBMC and neutrophils (Figure 5.5 A and B). Mean values for PBMC CR2 in controls was 79.81 ± 15.77 and that of patients was 46.15 ± 11.60 and mean values for neutrophil CR2 in controls was 83.76 ± 14.50 and that of patients was 52.76 ± 12.85 respectively.

Figure 5.5 (A and B): Levels of CR2 in PBMCs and Neutrophils determined by semi-quantitative RT-PCR. The plot shows the values of CR2 expression normalized by β-actin expression in controls (n=100) and RA patients (n=100). Each symbol represents one control (indigo triangles) or patient (pink stars), and the bar within each group represents the mean value. The P value was determined by Mann-Whitney test.
5.5.1 Correlation of CR2 with clinical parameters

The relationship of PBMC and neutrophil CR2 transcript levels with CIC, C3 and C3d were evaluated in both normal controls and patients.

5.5.1 (i) Correlations of PBMC and Neutrophil CR2 with CIC

In RA patients, CR2 transcript in PBMC positively correlated with CIC levels in controls ($p=0.0002$, $r=0.3611$, spearman rho analysis) and in patients there was no correlation ($p=0.3831$, $r=0.08816$, spearman rho analysis) (Figure 5.5.1 i A and B). However, there was no significant correlation between CR2 transcript in neutrophil with CIC levels in controls ($p=0.0915$, $r=0.1697$, spearman rho analysis) as well as in patients ($p=0.1180$, $r=0.1573$, spearman rho analysis) (Figure 5.5.1 i C and D).

![Correlation between CIC levels and CR2 PBMC transcript in normal controls and RA patients.](image1.png)

**Figure 5.5.1 (i) (A and B):** Correlation between CIC levels and CR2 PBMC transcript in normal controls and RA patients. Panels A and B represent correlation between CIC levels and CR2 PBMC transcripts in controls ($n=100$) and RA patients ($n=100$) respectively. The $P$ and $r$-values are indicated in the respective panels.

![Correlation between CIC levels and CR2 neutrophil transcript in normal controls and RA patients.](image2.png)

**Figure 5.5.1 (i) (C and D):** Correlation between CIC levels and CR2 neutrophil transcript in normal controls and RA patients. Panels C and D represent correlation between CIC levels and CR2 neutrophil transcripts in controls ($n=100$) and RA patients ($n=100$) respectively. The $P$ and $r$-values are indicated in the respective panels.
5.5.1 (ii) Correlations of PBMC and Neutrophil CR2 with C3

In RA patients, CR2 transcript in PBMC positively correlated with C3 levels in controls ($p=0.0134$, $r=0.2466$, spearman rho analysis) (Figure 5.5.1 ii A) as well as in patients ($p=0.0164$, $r=0.2396$, spearman rho analysis) (Figure 5.5.1 ii B). There is no significant correlation between CR2 transcript in neutrophils in controls ($p=0.3076$, $r=0.1031$, spearman rho analysis) (Figure 5.5.1 ii C) and it is positively correlated in patients ($p=0.0164$, $r=0.2396$, spearman rho analysis) (Figure 5.5.1 ii D).

**Figure 5.5.1 (ii) (A and B): Correlation between C3 levels and CR2 PBMC transcript in normal controls and RA patients.** Panels A and B represent correlation between C3 levels and CR2 PBMC transcripts in controls ($n=100$) and RA patients ($n=100$) respectively. The $P$ and $r$-values are indicated in the respective panels.

**Figure 5.5.1 (ii) (C and D): Correlation between C3 levels and CR2 neutrophil transcript in normal controls and RA patients.** Panels C and D represent correlation between C3 levels and CR2 neutrophil transcripts in controls ($n=100$) and RA patients ($n=100$) respectively. The $P$ and $r$-values are indicated in the respective panels.
5.5.1 (iii) Correlations of PBMC and Neutrophil CR2 with C3d

There is no significant correlation between CR2 transcript in PBMC in controls with C3d levels in controls (p=0.051, r=0.374, spearman rho analysis) (Figure 5.5.1 iii A) and it is positively correlated with patients (p=0.002, r=0.507, spearman rho analysis) (Figure 5.5.1 iii B). There is no significant correlation between CR2 transcript in neutrophils in controls (p=0.583, r=0.100, spearman rho analysis) (Figure 5.5.1 iii C) and it is positively correlated in patients (p=0.039, r=0.360, spearman rho analysis) (Figure 5.5.1 iii D).

Figure 5.5.1 (iii) (A and B): Correlation between C3d levels and CR2 PBMC transcript in normal controls and RA patients. Panels A and B represent correlation between C3 levels and CR2 PBMC transcripts in controls (n=32) and RA patients (n=33) respectively. The P and r-values are indicated in the respective panels.

Figure 5.5.1 (iii) (C and D): Correlation between C3d levels and CR2 neutrophil transcript in normal controls and RA patients. Panels C and D represent correlation between C3d levels and CR2 neutrophil transcripts in controls (n=32) and RA patients (n=33) respectively. The P and r-values are indicated in the respective panels.
5.6 Interrelationship between PBMC and Neutrophil transcripts of CR1 and CR2 among each other

5.6.1 Correlation of CR1 PBMC transcript with CR1 neutrophil transcript in both controls and patients

In controls no correlation was observed between CR1 PBMC transcript and in CR1 neutrophil transcript (p=0.092, r=0.169) (Figure 5.6.1 A), whereas in patients it shows a positive significant correlation (p=0.001, r=0.314) (Figure 5.6.1 B).

Figure 5.6.1 (A and B): Correlation between CR1 PBMC transcript and CR1 neutrophil transcript in both controls and patients. These panels represent correlation between CR1 PBMC transcript and CR1 neutrophil transcript in controls (n=100) and patients (n=100). The P and r-values are indicated in these panels.
5.6.2 Correlation of CR2 PBMC transcript with CR2 neutrophil transcript in both controls and patients

In both controls (p=0.0002, r=0.360) (Figure 5.6.2 A) and patients (p<0.0001, r=0.424) (Figure 5.6.2 B) significant positive correlation was observed between CR2 PBMC transcript and in CR2 neutrophil transcript.

![Graph showing correlation](image1)

**Figure 5.6.2 (A and B): Correlation between CR2 PBMC transcript and CR2 neutrophil transcript in both controls and patients.** These panels represent correlation between CR2 PBMC transcript and CR2 neutrophil transcript in controls (n=100) and patients (n=100). The P and r-values are indicated in these panels.

5.6.3 Correlation of CR1 PBMC transcript with CR2 PBMC transcript in both controls and patients

There was no correlation between CR1 and CR2 PBMC transcript in controls (p=0.877, r=0.015) (Figure 5.6.3 A), where as in patients it shows a positive significant correlation (p=0.010, r=0.253) (Figure 5.6.3 B).

![Graph showing correlation](image2)

**Figure 5.6.3 (A and B): Correlation between CR1 and CR2 PBMC transcript in both controls and patients.** These panels represent correlation between CR1 and CR2 PBMC transcript in controls (n=100) and patients (n=100). The P and r-values are indicated in these panels.
5.6.4 Correlation of CR1 neutrophil transcript with CR2 neutrophil transcript in both controls and patients

In both controls (p=0.005, r=0.276) (Figure 5.6.4 A) and patients (p<0.0001, r=0.458) (Figure 5.6.4 B) shows significant positive correlation between CR1 and CR2 neutrophil transcript.

Figure 5.6.4 (A and B): Correlation between CR1 and CR2 neutrophil transcript in both controls and patients. These panels represent correlation between CR1 and CR2 neutrophil transcript in controls (n=100) and patients (n=100). The P and r-values are indicated in these panels.
Results

5.7 Surface Protein Expression of Complement Receptor 1 and 2 (CR1 and CR2) in Controls and in RA Patients

5.7.1 Cell surface expression of CR1

Lymphocytes, monocytes and neutrophils were stained with monoclonal mouse anti-human CR1 antibody (serotec) and corresponding isotype control followed by incubation with FITC conjugated secondary antibody. The level of complement receptor 1 (CR1) in all these above cells were determined in 100 controls and 100 RA patients by flow cytometer.

In controls, the mean MFI (Mean Fluorescence Intensity) values for lymphocytes, monocytes and neutrophils were 1318 ± 423.6, 1517 ± 469 and 2176 ± 699.1 respectively. In patients, the mean values of CR1 for lymphocytes, monocytes and neutrophils were 768.6 ± 324.8, 1025 ± 396.2 and 1256 ± 465.8 respectively. In patients, surface expression of CR1 declined significantly on all the cells (p<0.0001, Mann-Whitney test) (Figure 5.7.1).

![Figure 5.7.1: Distribution of CR1 on leucocyte populations. Bar plot showing the expression of CR1 in terms of MFI for lymphocytes, monocytes and neutrophils in controls (n=100) and RA patients (n=100). The values are expressed in terms of Mean ± SD. The P value was calculated by Mann-Whitney test.](image)

5.7.1 (i) Expression of CR1 on Lymphocytes

The expression of CR1 in lymphocytes are significantly declined (according to the mean value of MFI) by 41.6% in RA patients when compared to controls (p<0.0001, Mann-Whitney test) (Figure 5.7.1 i D). The mean MFI values for CR1 in controls were 1318 ± 423.6 and that of patients was 768.6 ± 324.8 respectively.
Results

Figure 5.7.1(i) (A): Dot blot of Lymphocytes and Monocytes in FACS analysis

Figure 5.7.1(i) (B): Expression of complement receptor 1 in Lymphocytes in unstained cells; Figure 5.7.1(i) (C): Expression of complement receptor 1 in Lymphocytes in stained cells.

Figure 5.7.1(i) (D): Levels of CR1 in lymphocyte determined by flow cytometer. The plot shows the values of CR1 expression in controls (n=100) and RA patients (n=100). Each symbol represents one control (indigo triangles) or patient (pink stars), and the bar within each group represents the mean value. The P value was determined by Mann-Whitney test.
5.7.1 (ii) Expression of CR1 on Monocytes

The expression of CR1 in monocytes were significantly declined (according to the mean value of MFI) by 32.4% in RA patients when compared to controls (p<0.0001, Mann-Whitney test) (Figure 5.7.1 (ii) C). The mean MFI values for CR1 in controls were 1517 ± 469 and that of patients was 1025 ± 396.2 respectively.

**Figure 5.7.1(ii) (A):** Expression of complement receptor 1 in Monocytes in unstained cells; **Figure 5.7.1 (ii) (B):** Expression of complement receptor 1 in Monocytes in stained cells.

**Figure 5.7.1 (ii) (C):** Levels of CR1 in monocytes determined by flow cytometer. The plot shows the values of CR1 expression in normals (n=100) and RA patients (n=100). Each symbol represents one control (indigo triangles) or patient (pink stars), and the bar within each group represents the mean value. The P value was determined by Mann-Whitney test.

5.7.1 (iii) Expression of CR1 on Neutrophils

The expression of CR1 in neutrophils were significantly declined (according to the mean value of MFI) by 42.2% in RA patients when compared to controls (p<0.0001, Mann-
Whitney test) (Figure 5.7.1(iii) D). The mean MFI values for CR1 in controls were 2176 ± 699.1 and that of patients was 1256 ± 465.8 respectively.

Figure 5.7.1 (iii) (A): Dot blots of Neutrophils in FACS analysis.

Figure 5.7.1 (iii) (B): Expression of complement receptor 1 in Neutrophils in unstained cells; Figure 5.7.1. (iii) (C): Expression of complement receptor 1 in Neutrophils in stained cells.

Figure 5.7.1 (iii) (D): Levels of CR1 in neutrophils determined by flow cytometer. The plot shows the values of CR1 expression in controls (n=100) and RA patients (n=100). Each symbol represents one control (indigo triangles) or patient (pink stars), and the bar within each group represents the mean value. The P value was determined by Mann-Whitney test.
5.7.2 Cell surface expression of CR2

In controls, the mean MFI values for lymphocytes, monocytes and neutrophils were 1384 ± 428.5, 1851 ± 679.7 and 3040 ± 914.8 respectively. In patients, the mean values of CR2 for lymphocytes, monocytes and neutrophils were 931.2 ± 317.5, 1373 ± 362.5 and 2000 ± 699.8 respectively. In patients, the surface expression of CR2 was significantly declined on all the cells (p<0.0001, Mann-Whitney test) (Figure 5.7.2).

Figure 5.7.2: Distribution of CR2 on leucocyte populations. Bar plot showing the expression of CR2 in terms of MFI for lymphocytes, monocytes and neutrophils in controls (n=100) and RA patients (n=100). The values are expressed in terms of Mean ± SD. The P value was calculated by Mann Whitney test.
5.7.2 (i) Expression of CR2 on Lymphocytes

The expression of CR2 in lymphocytes were significantly declined (according to the mean value of MFI) by 32.7% in RA patients when compared to controls (p<0.0001, Mann-Whitney test) (Figure 5.7.2 i C). The mean MFI values for CR2 in controls were 1384 ± 428.5 and that of patients was 931.2 ± 317.5 respectively.

Figure 5.7.2 (i) (A): Expression of CR2 Lymphocytes in unstained cells; Figure 5.7.2 (i) (B): Expression of CR2 Lymphocytes in stained cells.

Figure 5.7.2 (i) (C): Levels of CR2 in lymphocyte determined by flow cytometer. The plot shows the values of CR2 expression in normals (n=100) and RA patients (n=100). Each symbol represents one control (indigo triangles) or patient (pink stars), and the bar within each group represents the mean value. The P value was determined by Mann-Whitney test.
5.7.2 (ii) Expression of CR2 on Monocytes

The expression of CR2 in monocytes were significantly declined (according to the mean value of MFI) by 25.8% in RA patients when compared to controls (p<0.0001, Mann-Whitney test) (Figure 5.7.2 ii C). The mean MFI values for CR2 in controls were 1851 ± 679.7 and that of patients was 1373 ± 362.5 respectively.

![Graph showing expression of CR2 in monocytes](image)

**Figure 5.7.2 (ii) (A):** Expression of CR2 Monocytes in unstained cells; **Figure 5.7.2 (ii) (B):** Expression of CR2 Monocytes in stained cells.

![Graph showing levels of CR2 in monocytes](image)

**Figure 5.7.2 (ii) (C):** Levels of CR2 in monocytes determined by flow cytometer. The plot shows the values of CR2 expression in controls (n=100) and RA patients (n=100). Each symbol represents one control (indigo triangles) or patient (pink stars), and the bar within each group represents the mean value. The P value was determined by Mann-Whitney test.
5.7.2 (iii) Expression of CR2 on Neutrophils

The expression of CR2 in neutrophils were significantly declined (according to the mean value of MFI) by 34.2% in RA patients when compared to controls (p<0.0001, Mann-Whitney test) (Figure 5.7.2 iii C). The mean MFI values for CR2 in controls were 3040 ± 914.8 and that of patients was 2000 ± 699.8 respectively.

![Figure 5.7.2 (iii) (A): Expression of CR2 Neutrophils in unstained cells; Figure 5.7.2 (iii) (B): Expression of CR2 Neutrophils in stained cells.](image)

![Figure 5.7.2 (iii) (C): Levels of CR2 in neutrophils determined by flow cytometer. The plot shows the values of CR2 expression in controls (n=100) and RA patients (n=100). Each symbol represents one control (indigo triangles) or patient (pink stars), and the bar within each group represents the mean value. The P value was determined by Mann-Whitney test.](image)
Results

5.8 Correlations of CR1, CR2, CIC, C3 and C3d with Disease Activity Score (DAS28) in 0 months (naïve RA).

The relationship of CIC, C3, C3d, CR1 and CR2 transcript with DAS28 were evaluated only in patients.

5.8.1 Correlation of CR1 transcript with DAS28 score

There is no significant correlation between PBMC CR1 transcript (p=0.6261, r=-0.06593, spearman rho analysis) (Fig 5.8.1 A) and neutrophil transcript (p=0.4528, r=-0.1014, spearman rho analysis) (Fig 5.8.1 B) with the disease activity score (DAS28) in RA patients (n=57).

![Figure 5.8.1 (A and B): Correlations between CR1 PBMC transcript and neutrophil CR1 transcript with DAS28 in RA patients. Panels A and B represent correlation between CR1 PBMC transcripts and CR1 neutrophil transcripts in RA patients (n=57) with its disease activity score (DAS28) respectively. The P and r- values are indicated in the respective panels.](image-url)
5.8.2 Correlation of CR2 transcript with DAS28 score

There is no significant correlation between PBMC CR2 transcript (p=0.7737, r=0.0389, spearman rho analysis) (Fig 5.8.2 A) and neutrophil transcript (p=0.0872, r=0.2286, spearman rho analysis) (Fig 5.8.2 B) with the disease activity score (DAS28) in RA patients (n=57) respectively.

Figure 5.8.2 (A and B): Correlation between CR2 PBMC transcript and neutrophil CR2 transcript with DAS28 in RA patients. Panels A and B represent correlation between CR2 PBMC transcripts and CR2 neutrophil transcripts in RA patients (n=57) with its disease activity score (DAS28) respectively. The P and r-values are indicated in the respective panels.

5.8.3 Correlation of CIC with DAS28 score

Correlation of circulating immune complex with the disease activity score (DAS28) were studied in 57 RA patients. We found a significant negative correlation between CIC and DAS28 (p=0.0435, r=-0.2684, spearman rho analysis) (Fig 5.8.3).

Figure 5.8.3: Correlation between CIC levels and DAS28 in RA patients. This panel represents correlation between CIC levels and DAS28 score in RA patients (n=57). The P and r-values are indicated in the panel.
5.8.4 Correlation of C3 with DAS28 score

Correlation of C3 with the disease activity score (DAS28) were studied in 57 RA patients. We found no significant correlation between C3 and DAS28 (p=0.8730, r=-0.2165, spearman rho analysis) (Fig 5.8.4).

Figure 5.8.4: Correlation between C3 levels and DAS28 in RA patients. This panel represents correlation between C3 levels and DAS28 score in RA patients (n=57). The P and r-values are indicated in the panel.

5.8.5 Correlation of C3d with DAS28 score

Correlation of C3d with the disease activity score (DAS28) were studied in 33 RA patients. We found no significant correlation between C3d and DAS28 (p=0.370, r=-0.161, spearman rho analysis) (Fig 5.8.5).

Figure 5.8.5: Correlation between C3d levels and DAS28 in RA patients. This panel represents correlation between C3d levels and DAS28 score in RA patients (n=33). The P and r-values are indicated in the panel.
5.9 Correlations of CR1 and CR2 with Disease Activity Score (DAS28) in 3 and 6 months Follow-ups

5.9.1 Expression of CR1 in follow-up patients

Expression of PBMC CR1 transcript and Neutrophil CR1 transcript were estimated in 17 follow up patients with 3 and 6 months of treatment (Figure 5.9.1). It was observed in DAS28 score, there was no significant change between 0 months and 3 months follow up. However, there was a significant reduction in 6 months follow up (4.12 ± 0.783, p<0.0001, Mann-Whitney test) than in 0 months (5.64 ± 0.85). In both PBMC and Neutrophil CR1, there is no such difference between the expression levels in 0 months and 3 months but increased in 6 months follow-ups. The mean ± S.D were given in the following table (Table.16).

Table 16: Different parameters of CR1 in follow-up patients

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Months</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAS28</td>
<td>0</td>
<td>5.64 ± 0.85</td>
</tr>
<tr>
<td>DAS28</td>
<td>3</td>
<td>5.08 ± 1.03</td>
</tr>
<tr>
<td>DAS 28</td>
<td>6</td>
<td>4.12 ± 0.78</td>
</tr>
<tr>
<td>CR1 PBMC</td>
<td>0</td>
<td>54.83 ± 13.21</td>
</tr>
<tr>
<td>CR1 PBMC</td>
<td>3</td>
<td>52.60 ± 8.54</td>
</tr>
<tr>
<td>CR1 PBMC</td>
<td>6</td>
<td>69.10 ± 11.81</td>
</tr>
<tr>
<td>CR1 Neutrophil</td>
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<td>47.30 ± 11.62</td>
</tr>
<tr>
<td>CR1 Neutrophil</td>
<td>3</td>
<td>54.64 ± 10.62</td>
</tr>
<tr>
<td>CR1 Neutrophil</td>
<td>6</td>
<td>69.81 ± 7.52</td>
</tr>
</tbody>
</table>

Figure 5.9.1: Expression of PBMC, Neutrophil CR1 transcript and DAS28 score in 0, 3 and 6 months follow-up patients. All results are expressed as mean ± SD. The bar shows mean values with standard deviation.
5.9.2 Expression of PBMC CR1 transcript in 0, 3 and 6 months follow-up patients with DAS28 score in 0 months

Figure 5.9.2 (A, B and C): Correlation of DAS28 score in 0 months with CR1 PBMC transcript in 0, 3 and 6 months follow-up patients (n=17). The P and r-values are indicated in the respective panel.

5.9.3 Expression of PBMC CR1 transcripts in 0, 3 and 6 months follow-up patients with DAS28 score in 3 months

Figure 5.9.3 (A, B and C): Correlation of DAS28 score in 3 months with CR1 PBMC transcript in 0, 3 and 6 months follow-up patients (n=17). The P and r-values are indicated in the respective panel.

5.9.4 Expression of PBMC CR1 transcripts in 0, 3 and 6 months follow-up patients with DAS28 score in 6 months

Figure 5.9.4 (A, B and C): Correlation of DAS28 score in 6 months with CR1 PBMC transcript in 0, 3 and 6 months follow-up patients (n=17). The P and r-values are indicated in the respective panel.
5.9.5 Expression of Neutrophil CR1 transcripts in 0, 3 and 6 months follow-up patients with DAS28 score in 0 months

![Graph](image1)

Figure 5.9.5 (A, B and C): Correlation of DAS28 score in 0 months with CR1 Neutrophil transcript in 0, 3 and 6 months follow-up patients (n=17). The P and r-values are indicated in the respective panel.

5.9.6 Expression of Neutrophil CR1 transcripts in 0, 3 and 6 months follow-up patients with DAS28 score in 3 months

![Graph](image2)

Figure 5.9.6 (A, B and C): Correlation of DAS28 score in 3 months with CR1 Neutrophil transcript in 0, 3 and 6 months follow-up patients (n=17). The P and r-values are indicated in the respective panel.

5.9.7 Expression of Neutrophil CR1 transcripts in 0, 3 and 6 months follow-up patients with DAS28 score in 6 months

![Graph](image3)

Figure 5.9.7 (A, B and C): Correlation of DAS28 score in 6 months with CR1 Neutrophil transcript in 0, 3 and 6 months follow-up patients (n=17). The P and r-values are indicated in the respective panel.
5.9.8 Expression of CR2 in follow-up patients

Expression of PBMC CR2 transcript and Neutrophil CR2 transcript were estimated in 17 follow-up patients with 3 and 6 months of treatment (Figure 5.9.8). It was observed in DAS28 score, there was no significant change between 0 months and 3 months follow up patients. However, there was a significant reduction in 6 months follow up (4.12 ± 0.78, p<0.0001, Mann-Whitney test) than in 0 months (5.64 ± 0.85). In both PBMC and Neutrophil CR2, there is no difference between 0 months and 3 months but increased in 6 months follow-ups. The mean ± SD were given in the following table (Table. 17).

Table 17: Different parameters of CR2 in follow-up patients

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Months</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAS28</td>
<td>0</td>
<td>5.64 ± 0.85</td>
</tr>
<tr>
<td>DAS28</td>
<td>3</td>
<td>5.08 ± 1.03</td>
</tr>
<tr>
<td>DAS 28</td>
<td>6</td>
<td>4.12 ± 0.78</td>
</tr>
<tr>
<td>CR2 PBMC</td>
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<td>44.19 ± 10.14</td>
</tr>
<tr>
<td>CR2 PBMC</td>
<td>3</td>
<td>57.53 ± 12.06</td>
</tr>
<tr>
<td>CR2 PBMC</td>
<td>6</td>
<td>92.83 ± 12.99</td>
</tr>
<tr>
<td>CR2 Neutrophil</td>
<td>0</td>
<td>46.82 ± 13.23</td>
</tr>
<tr>
<td>CR2 Neutrophil</td>
<td>3</td>
<td>55.86 ± 10.41</td>
</tr>
<tr>
<td>CR2 Neutrophil</td>
<td>6</td>
<td>101.30 ± 10.003</td>
</tr>
</tbody>
</table>

Figure 5.9.8: Expression of PBMC, Neutrophil CR2 transcript and DAS28 score in 0, 3 and 6 months follow-up patients. All results are expressed as mean ± SD. The bar shows mean values with standard deviation.
5.9.9 Expression of PBMC CR2 transcripts in 0, 3 and 6 months follow-up patients with DAS28 score in 0 months

Figure 5.9.9 (A, B and C): Correlation of DAS28 score in 0 months with CR2 PBMC transcript in 0, 3 and 6 months follow-up patients (n=17). The P and r- values are indicated in the respective panel.

5.9.10 Expression of PBMC CR2 transcripts in 0, 3 and 6 months follow-up patients with DAS28 score in 3 months

Figure 5.9.10 (A, B and C): Correlation of DAS28 score in 3 months with CR2 PBMC transcript in 0, 3 and 6 months follow-up patients (n=17). The P and r- values are indicated in the respective panel.

5.9.11 Expression of PBMC CR2 transcripts in 0, 3 and 6 months follow-up patients with DAS28 score in 6 months

Figure 5.9.11 (A, B and C): Correlation of DAS28 score in 6 months with CR2 PBMC transcript in 0, 3 and 6 months follow-up patients (n=17). The P and r- values are indicated in the respective panel.
5.9.12 Expression of Neutrophil CR2 transcripts in 0, 3 and 6 months follow-up patients with DAS28 score in 0 months

Figure 5.9.12 (A, B and C): Correlation of DAS28 score in 0 months with CR2 Neutrophil transcript in 0, 3 and 6 months follow-up patients (n=17). The P and r-values are indicated in the respective panel.

5.9.13 Expression of Neutrophil CR2 transcripts in 0, 3 and 6 months follow-up patients with DAS28 score in 3 months

Figure 5.9.13 (A, B and C): Correlation of DAS28 score in 3 months with CR2 Neutrophil transcript in 0, 3 and 6 months follow-up patients (n=17). The P and r-values are indicated in the respective panel.

5.9.14 Expression of Neutrophil CR2 transcripts in 0, 3 and 6 months follow-up patients with DAS28 score in 6 months

Figure 5.9.14 (A, B and C): Correlation of DAS28 score in 6 months with CR2 Neutrophil transcript in 0, 3 and 6 months follow-up patients (n=17). The P and r-values are indicated in the respective panel.
Results

5.10 Correlation of CIC with Disease Activity Score (DAS 28) in 3 and 6 months Follow-ups

5.10.1 Correlation of CIC with disease activity score (DAS28) in 3 months follow-up patients

Correlation of circulating immune complex with the disease activity score (DAS28) after 3 months of treatment were studied in 17 RA patients. We found a significant positive correlation between CIC and DAS28 (p=0.0483, r=0.4853, spearman rho analysis) (Fig 5.10.1).

![Figure 5.10.1: Correlation between CIC levels and DAS28 in RA patients in 3 months follow-up. This panel represents correlation between CIC levels and DAS28 score in RA patients (n=17). The P and r-values are indicated in the panel.](image)

5.10.2 Correlation of CIC with disease activity score (DAS28) in 6 months follow-up patients

After 6 months of treatment, we found a significant positive correlation between CIC and DAS28 (p=0.0003, r=0.768, spearman rho analysis) (Fig 5.10.2).

![Figure 5.10.2: Correlation between CIC levels and DAS28 in RA patients in 6 months follow-up. This panel represents correlation between CIC levels and DAS28 score in RA patients (n=17). The P and r-values are indicated in the panel.](image)
5.11 Correlation of C3 with Disease Activity Score (DAS 28) in 3 and 6 months Follow-ups

5.11.1 Correlation of C3 with disease activity score (DAS28) in 3 months follow-up patients

Correlation of C3 with the disease activity score (DAS28) after 3 months of treatment were studied in 17 RA patients. We found no significant correlation between C3 and DAS28 (p=0.4623, r=-0.1912, spearman rho analysis) (Fig 5.11.1).

Figure 5.11.1: Correlation between C3 levels and DAS28 in RA patients in 3 months follow-up. This panel represents correlation between C3 levels and DAS28 score in RA patients (n=17). The P and r-values are indicated in the panel.

5.11.2 Correlation of C3 with disease activity score (DAS28) in 6 months follow-up patients

We found a positive significant correlation between C3 and DAS28 (p=0.009, r=0.612, spearman rho analysis) (Fig 5.11.2) in 6 months of follow up study in RA patients.

Figure 5.11.2: Correlation between C3 levels and DAS28 in RA patients in 6 months follow-up. This panel represents correlation between C3 levels and DAS28 score in RA patients (n=17). The P and r-values are indicated in the panel.
5.12 Correlation of C3d with Disease Activity Score (DAS 28) in 3 and 6 months Follow-ups

5.12.1 Correlation of C3d with disease activity score (DAS28) in 3 months follow-up patients

Correlation of C3d with the disease activity score (DAS28) after 3 months of treatment were studied in 15 RA patients. We found a positive significant correlation between C3 and DAS28 (p=0.042, r=0.528, spearman rho analysis) (Fig 5.12.1).

![Figure 5.12.1: Correlation between C3d levels and DAS28 in RA patients in 3 months follow-up. This panel represents correlation between C3d levels and DAS28 score in RA patients (n=15). The P and r-values are indicated in the panel.](image)

5.12.2 Correlation of C3d with disease activity score (DAS28) in 6 months follow-up patients

We found a positive significant correlation between C3d and DAS28 (p=0.014, r=0.614, spearman rho analysis) (Fig 5.12.2) in 6 months of follow up study in RA patients.

![Figure 5.12.2: Correlation between C3d levels and DAS28 in RA patients in 6 months follow-up. This panel represents correlation between C3d levels and DAS28 score in RA patients (n=15). The P and r-values are indicated in the panel.](image)
6. DISCUSSION

The complement system has been implicated in the pathology of Rheumatoid arthritis. A vast body of literature supports the protective role of the proteins of the complement cascade against the initiation of the autoimmune disease through its role in solubilizing immune complex, clearing apoptotic bodies from the circulation and inducing tolerance through its function of negative selection of self-reacting B cells (Pickering et al., 2000; Walport, 2002). An equally impressive array of studies point out the deleterious role of the cascade proteins in bringing about tissue injury and inflammation in the effector phase of the disease (Lachmann et al., 1962; Gawryl et al., 1988).

Decrease of CR1 numbers on erythrocytes, (Miyakawa et al., 1981; Iida et al., 1982; Wilson et al., 1982) glomerular podocytes, B cells and PMN (Wilson et al., 1986; Marquart et al., 1995) as well as defective complement mediated phagocytosis or immune clearance (Arora et al., 2000b) had been reported in RA and SLE (Arora et al., 2000b). Modulation of leucocyte CR1 (L-CR1) in SLE has been reported (Arora et al., 2004, 2007). Decreased level of CR2 in peripheral blood B-cell upon activation had also been reported (Roome and Reading, 1987). The reduced expression of CR1 and CR2 on the B cells of RA patients is known for long, their exact role in B-cell tolerance and autoimmunity is not yet fully understood (Kremlitzka et al., 2012). Reduced cell surface expression of the CR2 by synovial fluid B and T lymphocytes had been observed in RA patients (Illges et al., 2000). The reason for lower than normal CR1 expression in this disease and several other inflammatory conditions is not clear. We aimed at studying the modulation of leucocyte CR1 and CR2 transcript and cell surface expression in patients with rheumatoid arthritis and compared that with the controls. Study involved the retrospective study in patients who had not started with DMARD (Disease Modifying Anti Rheumati Drug) and their prospective follow ups and their cause on treatment. Levels of CR1 and CR2 were evaluated and their correlations with different disease activity were determined.

One hundred controls and 100 patients with RA were investigated. Healthy volunteers, with no history of autoimmune disorders, major infection and, other inflammatory diseases were recruited as controls. Among 100 control subjects, 86 were female and 14 were male (age18 - 41 yrs). The patients were selected from the OPD of Medicine Department, All India Institute of Medical Sciences, New Delhi. The group consisted of 94 females and 6 males (18 - 52yrs). Of these one hundred patients, 17 patients were enrolled for a longitudinal follow up study. Blood from these patients were collected on
CR1 and CR2 expression were studied in terms of the levels of transcript and surface protein. Levels of CIC, C3 and C3d were determined in the patients and controls. In addition, a longitudinal follow up study of the patients was also carried out. Levels of CR1 and CR2 transcript were determined by semi quantitative RT-PCR. Surface protein levels in CR1 and CR2 were determined by flow cytometry. C3, C3d, CIC and DAS28 were determined to assess the clinical correlations of CR1 and CR2 expression. For modulation of E-CR1, the suggested mechanism for long had been its shedding off from these cells by enhanced proteolytic cleavage in aforesaid disease condition. Genetic inheritance was also suggested. There was no information available on the state of CR1 expression and the underlying mechanisms. Our lab had investigated on the subject and it was found that leucocyte CR1 expression in SLE was modulated at the levels of gene transcription and translation to membrane and enhanced proteolytic shedding from the cells. The same study for CR1 and CR2 in RA is not known. Therefore, this study aimed at elucidating the expression of CR1 and CR2 at the transcript and protein levels and determines their correlations with the disease activity of RA.

The study commenced with an extensive standardization of the methods to determine on the optimal/appropriate experimental conditions and protocols. This was done to ensure the accuracy and reliability of the findings. The level of CR1 in both cell surface and transcript levels were determined in 100 controls and 100 RA patients.

6.1 Expression of CR1 Transcript in Controls and Patients with RA

Level of leucocyte CR1 transcript was determined by semi-quantitative RT-PCR. In semi-quantitative RT-PCR, the comparisons between the samples are expressed as percent difference relative to the internal control. We performed semi-quantitative RT-PCR over real-time because of certain advantages. It is cost effective and does not require any specialized equipment. Gasque and Morgan, in 1996, followed the semi-quantitative RT-PCR method to analyze the CR1 transcript expression in oligodendrocytes. The standard method was followed including RNA isolation, cDNA production and PCR amplification. Purity of RNA was checked by the ratio of A_{260}/A_{280} and quantify by using nanodrop. The RNA samples having ratio between 1.9 - 2.2 and intact RNA bands (28s:18s, 2:1) were taken for reverse transcription. The reverse transcription was done according to the standard method using RT enzyme and random
decamers and quantified by densitometry. The sequences of gene-specific forward and reverse primers for CR1 (Arora et al., 2004) and β-actin (Tripathi et al., 2009) were taken from the published report. PCR cycles for CR1 were previously standardized in our lab (Arora et al., 2004). However, needful modifications were done.

In this study we found significant decline in the levels of CR1 transcript in both PBMC and Neutrophils from RA patients as compared to controls. The p values were assessed by Mann-Whitney test (Figure 5.4 A and B). Complement receptor 1 is a protein of immense importance in protecting the self-tissue against complement driven injury and subsequent manifestations (Arora et al., 2004). The protein is also important in the regulation of T and B cell responses, immune tolerance and direct host pathogen interactions (Fearon, 2000; Carroll, 2004; Isaac et al., 2009; Mihlan et al., 2011). A study from India by Arora et al., 2000b showed marked decline in the levels lower than normal levels of E-CR1 in patients with active SLE and RA. The levels, however, increased with the improvement of the health condition of the patients. The levels of CR1 further declined in those patients who either did not respond positively to the medicines or discontinued with the treatment. These patients had the worst prognosis. These observations suggested CR1 as a putative disease activity marker (Arora et al., 2004; Sivasankar et al., 2004). Another study showed confirmation of L-CR1 transcript as a prognostic marker for SLE (Arora et al., 2011). The level of N-CR1 is significantly higher in active RA patients (Paoliello-Paschoalatoa et al., 2011).

6.1.1 Correlations of CR1 transcript with clinical parameter (CIC, C3 and C3d)

Levels of CIC, C3 and C3d were measured in controls and RA patients and their correlations with DAS28 score were calculated. CIC is one of the most important pathogenic factors in the disease manifestations of RA. The levels of circulating immune complex (CIC) were measured in all 100 controls and 100 RA patients. The levels of CIC increased significantly by 52.1% in RA patients as compared to controls (p<0.0001, Mann-Whitney test) (Figure 5.3.1). In RA patients, CR1 transcript in PBMC showed significant negative correlation with CIC levels in controls and in patients. However, there was no significant correlation between CR1 transcript in neutrophil with CIC levels in controls as well as in patients (Figure 5.4.1 i A, B, C and D). Both CR1 and immune complex suppress the antibody production by B-cells. Lower levels of CR1 in B-cells in reciprocal relation with CIC may suggest impaired regulatory function of CR1 and increased production of autoantibodies and thus immune complexes. Correlations of circulating immune complex with the disease activity score (DAS28) were studied in 57 RA patients. We
found a significant negative correlation between CIC and DAS28 (Figure 5.8.3). Studies on the patients with RA and kidney disorders also showed a similar correlation between E-CR1 and CIC (Sivasankar et al., 2004). While IC overload may primarily be due to excessive formation of auto-antibodies, its defective clearance may propagate a vicious cycle. E-CR1 is long identified as a vehicle for immune complex clearance. CR1 plays an important role in the clearance of C3b and C4b- opsonized immune complexes that are transported to the liver for degradation or to the spleen for antigen presentation. On monocytes, the receptor induces phagocytosis and is involved in antigen presentation of complement-coupled particles (Nilsson et al., 2009). Arora et al., 2000a earlier suggested an important role of CR1 in uptake of CIC by phagocytic cells independently or in synergism with Fc receptors. The negative correlation between PBMC CR1 and CIC as observed by us suggests an important role of CR1 in immune complex clearance in addition to that by E-CR1. We measured IC as a whole by PEG precipitation and therefore could not differentiate the size or the class of the constituent antibody.

CIC causes enhanced activation of the complement cascade in the plasma. Exaggerated complement consumption leading to the deficiency of complement components is one of the major consequences of immune complex overload (Schifferli and Taylor, 1989). Since C3 is the merging point in the activation of complement cascade by different pathways, C3 had been the molecule of focus in different investigations carried out on autoimmune disorder. In our study, the levels of C3 were estimated in 100 controls and 100 RA patients by nephelometry in the serum samples. We found a significant \((p<0.0001, \text{Mann-Whitney test})\) increase in 93.7\% in the level of C3 in RA patients (Figure 5.3.2). Moreover, in RA patients, a significant negative correlation between PBMC CR1 transcripts with C3 levels in controls was observed. However, there was no significant correlation between PBMC CR1 transcripts in with C3 levels in patients. There was no significant correlation between CR1 transcript in neutrophils in both control and in patients (Figure 5.4.1 ii A, B, C and D). Correlation of C3 with the disease activity score (DAS28) was studied in 57 RA patients. We found no significant correlation between C3 and DAS28 (Figure 5.8.4). The increased consumption of C3 in patients may be due to the lowering of the levels of CR1 in patients. Although a similar proposition had been made for E-CR1 in relation to C3 levels in autoimmune disorders, our findings suggest an intimate relationship between the levels of C3 and CR1. C3 level was determined by a number of factors which include rate of synthesis determined partly by genetic factors (Alper and Rosen, 1967) rate of consumption (Hunsicker et al., 1972) and compensatory increase in the synthesis of C3 in inflammation which can mask increased consumption.
Moreover, localized synthesis of C proteins and restricted activation of the complement cascade in inflamed tissues is not reflected by a decreased level of plasma C3 as exemplified by diseases like myasthenia gravis or membranous nephritis (Ashizawa and Appel, 1985; Cavallo, 1994; Couser, 1999). Hence it was not unexpected that C3 level is not associated with DAS28 score.

The level of C3d largely reflects the extent of C3 activation. The quantitation of C3d levels has allowed for an estimation of complement activation in patients with RA patients. C3d has been argued to be a better marker for SLE disease activity because C3d is a direct product of the complement activation unlike C3, which is a substrate of C activation. Morrow et al., 1983 shown that C3d levels were raised above normal in both SLE and RA patients, presumably affecting the inflammatory processes involved in these diseases. C3d levels were associated with the severity of disease in RA and proved to be a much better indicator than C3 and plasma C3d levels correlated with clinical activity (Mallya et al., 1982). It has been reported that C3d levels raised in synovial fluid from RA patients (Perrin et al., 1975). The level of C3d was estimated in 32 controls and 33 RA patients by ELISA kit. We found a significant (p<0.0001, Mann-Whitney test) increase in 49.4% in the level of C3d in RA patients (Figure 5.3.3). In RA patients, a significant positive correlation between PBMC CR1 transcripts with C3d levels in controls and patients were observed (5.4.1 iii A and B). There was a significant positive correlation between CR1 transcript in neutrophils in both control and in patients (Figure 5.4.1 iii C and D). Correlation of C3d with the disease activity score (DAS28) was studied in 33 RA patients. We found no significant correlation between C3d and DAS28 (Figure 5.8.5). This cumulative findings observed in our patients is disease acquired may be due to exaggerated activation of complement and dysregulation of the complement cascade by CIC and L-CR1 respectively. The phenomenon is interlinked and overlapping. There are several reports on the merit of C3d measurement to predict prognosis of SLE, the role of C3d as a SLE disease marker is inconclusive. Negi et al., 2000 reported that though the level of C3d in the plasma of SLE patients is significantly higher than that of normal controls, the level was similar in active and inactive disease. According to a report by Senaldi et al., 1988, C3d levels were elevated but did not linearly correlate with disease activity.

There is no significant correlation between PBMC CR1 transcript and neutrophil transcript with the disease activity score (DAS28) in RA patients (Figure 5.8.1 A and B). CR1 is known to be modulated in auto-immune, inflammatory and infectious diseases. A correlation of the levels of CR1 on erythrocytes and glomerular podocytes with the
disease activity, severity and prognosis in auto-immune disorders like glomerulonephritis and SLE has been demonstrated (Mitchell et al., 1989; Sivasankar et al., 2004; Birmingham et al., 2006). CR1 expression declines in these diseases. The down regulation of CR1 expression in these diseases is closely related to disease pathology. However, very little information is available on the factors and mechanisms that may be involved in the regulation of CR1 expression. A prognostic significance for urinary CR1 and E-CR1 is envisaged (Sivasankar et al., 2004). Simultaneously, soluble recombinant CR1 is found effective in the treatment of inflammatory disease including GN and MI (Weisman et al., 1990; Couser, 1999). It has been under active investigation to elucidate the mechanisms that may underlay the deficient CR1 expression in these diseases.

6.2 Expression of CR2 Transcript in Controls and Patients with RA

Level of leucocyte CR2 transcript was determined by semi-quantitative RT- PCR. The PCR protocol for CR2 was extensively standardized. The sequences of gene-specific forward and reverse primers for CR2 (Chang et al., 2005) was taken from the published report. We performed a series of trial for CR2 standardization. The first step was to choose an optimum annealing temperature for efficient PCR reaction. Annealing of fragments is usually carried out 3-5°C lower than the melting temperature ($T_m$; salt adjusted method) of the forward and reverse primers. If the annealing temperature is too low, non-specific annealing of primers may occur, resulting in the amplification of unwanted fragments. PCR amplification of the CR2 gene with specific primer pair yields a 150bp product. Various primer concentrations (3.12 ,6.25 ,12.5 and 25 pico moles) and annealing temperature (gradient PCR -57°C, 58°C, 58.7°C, 59.5°C, 60.3°C, 61.1°C, 62.5°C, 63°C) were used to determine the optimum PCR conditions. cDNA concentration and PCR cycle was same as that used for RT- PCR for CR1. 1µl cDNA, 59.5°C annealing temperature and 12.5  picomoles primer concentrations were found to be optimum as they gave specific amplification of the target band with no non - specificity. Hence these conditions were used for further RT- PCR. Levels of transcript were expressed as percent difference relative to the control sample, giving semi-quantitative results. To minimize the sample to sample variation, internal controls such as β-actin was used (Suzuki et al., 2003). In this study, we found that the expressions of CR2 transcript were reduced significantly in both PBMC and Neutrophils in RA patients as compared to controls. The p values were assessed by Mann-Whitney test (Figure 5.5 A and B). To the best of our knowledge, much work is not carried out on this receptor in India. Studies shows that decreased level of CR2 in peripheral blood B-cell upon activation (Roome and Reading, 1987) and reduced expression of the CR2 by synovial fluid B and T
lymphocytes in RA patients (Illges et al., 2000). Decreased CR2 expression is due to increased shedding of the receptor, however, RA patients shed the same amounts of CR2 as healthy individuals and they even display reduced levels of soluble CR2. Interestingly, synovial B cells express even less CR2 compared to peripheral blood B cells in RA patients (Prokopec et al., 2010).

### 6.2.1 Correlations of CR2 transcript with clinical parameter (CIC, C3 and C3d)

We found a significant positive correlation between CR2 PBMC transcripts with CIC levels in controls and no correlation in RA patients. There was no significant correlation between CR2 transcript in neutrophil with CIC levels in controls as well as in patients (Figure 5.5.1 i A, B, C and D). There was a significant positive correlation between CR2 PBMC transcript with C3 levels in both controls and in patients. There was a significant positive correlation between CR2 neutrophil transcripts with C3 levels in RA patients and no correlation with the controls (Figure 5.5.1 ii A, B, C and D). The interaction of C3d with complement receptor 2 (CR2), plays an important role in B cell activation and maturation. There was no significant correlation between CR2 PBMC transcript and CR2 neutrophil transcript with C3d levels in controls (Figure 5.5.1 iii A and C). However, there was a significant positive correlation between CR2 PBMC transcript and CR2 neutrophil transcript with C3d levels in patients (Figure 5.5.1 iii B and D). This may indicates the reactive increase in production of these proteins rather than their consumption. There is no significant correlation between PBMC CR2 transcript and neutrophil transcript with the disease activity score (DAS28) in RA patients (Figure 5.8.2 A and B). CR2 has been shown to play a critical role in humoral immune responses to T-dependent Ags and thereby acts as a bridge between innate and adaptive immunity. Expression of CR2 on both B cells as well as FDC is critical to its role in T-dependent Ag responses (Myones and Ross, 1987). This immune enhancing activity of CR2 has been used to create highly immunogenic C3d-bound Ags that generate robust humoral responses. CR2 participate in the generation of a normal immune response by internalizing and directing C3-bound Ag into the class II processing pathway of B cells (Cherukuri et al., 2001b). It is also shown to have a direct influence on B cell-T cell signal exchange by simultaneous up-regulation of CD80 and CD86 on murine splenic B cells (Kozono et al., 1998). The other functions of CD21 though not yet clear are in development and maintenance of B1 cells (Ahearn et al., 1996). In human pro- and pre-B cells the expression of the CD21 gene is silenced by methylation of a CpG island in its promoter. Expression in mature B cells is accompanied by the loss of CpG-methylation (Schwab and Illges, 2001a,
C3 deposition on B cells may enhance their interaction with CD21 on follicular dendritic cells (FDC) and vice versa.

### 6.3 Follow-up Studies

The level of cell surface protein may not give the true level of the total protein synthesized at a given point of time since the expressed protein may be distributed in other cellular compartments and also may be secreted out of the cell. In soluble forms these proteins are present in plasma, tears, seminal fluids, urine and various other body fluids. We therefore focused to mRNA levels of CR1 and CR2 for different aspects of our follow up studies. Existing literature and recent studies from our lab has shown decreased level of CR1 expression at the transcript level in leucocytes from SLE patients. The follow up studies bring the most conclusive evidence in understanding of the role of a factor as a determinant of the disease pathology and course of the disease. Many patients with poor prognosis do not attend the routine clinics and many patients who initially volunteered to participate in the investigation refused to continue further. These are some of the reasons why a systematic follow up of RA is difficult. We could study 17 volunteers systematically at 0 day, 3 months and 6 months. The studies which were followed up beyond this period could not be systematized. Levels of several inflammatory proteins including complement peptides showed altered patterns during the course of the disease especially during the flare and remission (Ross et al., 1985; Holme et al., 1986; Thomsen et al., 1987). One of the previous studies conducted in this lab showed that the levels of complement regulatory proteins including E-CR1 declined in the active stages of RA and increased significantly in the patients who entered remission (Arora et al., 2001). Expression of PBMC CR1 transcript and Neutrophil CR1 transcript was estimated in the 17 follow up patients with 3 and 6 months of treatment. It was observed in DAS28 score, there was no significant change between 0 months and 3 months follow up. However, there was a significant reduction in 6 months follow up than in 0 months. In both PBMC and Neutrophil CR1, there was no such difference between 0 months and 3 months but increased in 6 months follow-ups (Figure 5.9.1 to 5.9.7).

Expression of PBMC CR2 transcript and Neutrophil CR2 transcript was estimated in the 17 follow up patients with 3 and 6 months of treatment. It was observed in DAS28 score, there was no significant change between 0 months and 3 months follow up. However, there was a significant reduction in 6 months follow up than in 0 months. In both PBMC and Neutrophil CR2, there was no difference between 0 months and 3 months but increased in 6 months follow-ups (Figure 5.9.8 to 5.9.14).
Correlation of circulating immune complex with the disease activity score (DAS28) after 3 and 6 months of treatment were studied in 17 RA patients. We found a significant positive correlation between CIC and DAS28 after both 3 and 6 months of treatment (Figure 5.10.1 and 5.10.2). Correlation of C3 with the disease activity score (DAS28) after 3 and 6 months of treatment were studied in 17 RA patients. We found no significant correlation between C3 and DAS28 after 3 months of treatment but there was a significant positive correlation found between C3 and DAS28 in 6 months of follow up study in RA patients (Figure 5.11.1 and 5.11.2). Correlation of C3d with the disease activity score (DAS28) after 3 and 6 months of treatment were studied in 15 RA patients. We found a significant positive correlation between C3d and DAS28 in both 3 and 6 months of follow up study in RA patients (Figure 5.12.1 and 5.12.2).

Thus, our follow up studies clearly showed a relationship between the up-regulation of the CR1 and CR2 levels and their importance as putative disease marker.

**6.4 Surface Protein Expression of CR1 in Controls and Patients with RA**

Levels of cell surface protein were determined by flow cytometry. The results were expressed in terms of mean fluorescence intensity (MFI). Non-specific staining was ruled out by running a parallel set of cells stained with isotype antibody for each experiment. For the stained cells, a shift in CR1 peak with respect to the isotype control in (a) Lymphocytes (b) Monocytes and (c) Neutrophils were observed. Our study showed that the normal distribution of CR1 levels was in the order of Neutrophils > monocytes > lymphocytes. This pattern though was maintained in the patients, all these cell populations showed decline in the levels of CR1 as compared to the respective cell populations of the controls. The surface expression of CR1 was significantly declined (according to the mean value of MFI) in lymphocytes (41.6%), monocytes (32.4%) and in neutrophils (42.2%) in RA patients as compared to controls. The p values were assessed by Mann-Whitney test (Figure 5.7.1). Several studies (Miyakawa et al., 1981; Ross et al., 1985; Thomsen et al., 1987) including those carried out in our lab (Kumar et al., 1992; Arora et al., 2000, 2001; Raju et al., 2001; Katyal et al., 2004; Sivasankar et al., 2004) had documented lower than normal levels of E-CR1 in the patients with glomerulonephritis (GN), Systemic Lupus Erythematosus (SLE) and Rheumatoid Arthritis (RA). Deficiency of CR1 in these diseases is speculated as a major cause for IC non clearance and exaggerated C-activation in these diseases.
6.5 Surface Protein Expression of CR2 in Controls and Patients with RA

The surface expression of CR2 by flow cytometry was done similarly as CR1 which was discussed in the previous section. Non-specific staining for CR2 was ruled out by running a parallel set of cells stained with isotype antibody for each experiment. For the stained cells, a shift in CR2 peak with respect to the isotype control in (a) Lymphocytes (b) Monocytes and (c) Neutrophils were observed. Our study showed that the normal distribution of CR2 levels was in the order of Neutrophils > monocytes > lymphocytes. This pattern though was maintained in the patients, all these cell populations showed decline in the levels of CR2 as compared to the respective cell populations of the normals. *The surface expression of CR2 were significantly declined (according to the mean value of MFI) in lymphocytes (32.7%), monocytes (25.8%) and in neutrophils (34.2%) in RA patients as compared to controls. The p values were assessed by Mann-Whitney test* (Figure 5.7.2). CR2 in FDC plays a very important role in rescuing antigen-activated B cells from apoptosis (Liu et al., 1989), promotion of somatic hypermutation (Apel and Berek, 1990; Nie et al., 1997) and class switch (Croix et al., 1996; Wu et al., 1996). In RA, all patients develop autoimmunity, whether or not they display CD21/B cells, and it is unclear how these B cells contribute to the initiation and progression of the disease (Menard et al., 2011).

6.6 Correlations between Complement Receptor 1 and Complement Receptor 2

Both CR1 and CR2 are expressed by mature B cells (Wilson et al., 1986). The mechanism of complement regulation of the B cells response appears to involve the interaction of the complement activation products (C3b and C3d) and CR1 and CR2 on the cell surfaces, increasingly the retention of antigen in lymphoid tissue and enhancing the B cell response. Under many conditions, CR1 and CR2 levels on B-lymphocytes as well as CR1 on leucocytes and erythrocytes are reduced (Benedetto et al., 1992; Ross et al., 1985) in fact soluble CR2 is found in the circulation in normal individuals (Huemer et al., 1993; Fremeaux-Bacchi et al., 1996, 1999) and at increased levels in certain diseases. CR2 can be shed and or reduced in copy number on B or T cells and one or more undefined proteases may cut CR2 (Fremeaux-Bacchi et al., 1996, 1999). RA patients had significantly reduced expression of B cells compared with healthy controls (Nilsson et al., 2009). Further, B cells from RA patients expressed markedly lower levels of CR1 and CR2. It has been observed, both in human and mice show that CR1 and CR2 play a role in arthritis susceptibility, while the severity of disease might not be directly linked to these receptors. *This is supported by our findings in humans were RA patients had lower levels of...*
CR1 and CR2 than healthy individuals and no difference was seen in the receptor levels between patients with an active disease compared to patients with a quiescent disease. Thus, this suggests that CR1 and CR2 receptor expression is not associated with the severity of arthritis but rather associated with disease development. Our study shows that in controls there was no correlation between CR1 PBMC transcript and in CR1 neutrophil transcript, whereas in patients it shows a positive significant correlation (Figure 5.6.1 A and B). There was a significant positive correlation between CR2 PBMC transcript and CR2 neutrophil transcript in both controls and patients (Figure 5.6.2 A and B). There was no correlation between CR1 and CR2 PBMC transcript in controls, whereas in patients it shows a positive significant correlation (Figure 5.6.3 A and B). A significant positive correlation was found between CR1 and CR2 neutrophil transcripts in both controls and patient (Figure 5.6.4 A and B).

The levels of CR1 and CR2 transcripts and proteins in patients remained markedly low as compared to the controls throughout the course of the study. In essence, our findings suggest a close relationship of CR1 and CR2 with the pathophysiology and disease activity of RA. The findings also may have important diagnostic, prognostic and therapeutic implications.
7. SUMMARY OF THE WORK AND CONCLUSIONS

RA is a chronic, progressive, systemic autoimmune disorder. The disease affects multiple organs, including the joints, skin, heart, lungs and eyes. It is also the most common form of inflammatory arthritis in adults, affecting approximately 0.3 -1.2 % of world’s population and 0.75% of India’s population. Till date, no specific disease activity marker is at sight and disease etiology remains unknown. An in depth study of various components of the innate immunity, acquired immunity and other molecular factors is warranted to gain an insight into various aspects of the disease. The advancing literature suggests that the expression levels of complement receptor 1 and 2 (CR1 and CR2) were pivotal in the association, pathogenesis and prognosis of RA. Available information of the involvement of these receptors in RA is largely based on animal experimentations. Studies on human subjects are essential to gain insight into the role of these proteins in humans suffering from RA. With the above notion and background information, we aimed at elucidating the expression of complement receptors 1 and 2 in relation to pathophysiology and severity of RA.

7.1 Aim and Objectives

The investigation aimed at studying the **Expression levels of CR1 and CR2 on the lymphocytes, monocytes and neutrophils in the healthy individuals and its relationship with the pathophysiology and clinical disease activity of Rheumatoid Arthritis.** The objectives were to: **study** and compare the expression profile of the complement receptors CR1 and CR2 in three different blood cells i.e. lymphocytes, monocytes and neutrophils at cell surface and transcript levels in controls and patients suffering from Rheumatoid Arthritis, **to study** the levels of C3, C3d and CIC in stored plasma and serum samples, relate that with both the cell surface and transcript levels of CR1 and CR2 and **to evaluate** the correlation among the above parameters and its relationship with the clinical disease activity.

7.2 Study Subjects

We studied 100 controls and 100 patients with RA to achieve the aim and objectives stated above. The patients were selected from the OPD of Medicine Department, All India Institute of Medical Sciences, New Delhi. Of these one hundred patients, 17 patients were enrolled for a longitudinal follow up study. Blood from these patients were collected on the day 0 (1st day of diagnosis, treatment naïve) and at the end of the 3rd month and 6th month of treatment. The patients were categorized as follows:
Group - 1: The patients who are coming for the first time to OPD without any medication (n=100).
Group - 2: 17 patients were enrolled for longitudinal follow up studies.

Clinical details of the patients were obtained from hospital records and DAS28 score was calculated accordingly. Out of 100 RA patients we calculated, DAS28 score available only for 57 patients. Patients were diagnosed in the department of medicine, AIIMS and their suitability for the study was determined with the help of clinicians. Ethical clearance was obtained from the ethical committee of the All India Institute of Medical Sciences before study.

7.3 Methodology and Study Design

CR1 and CR2 expression were studied in terms of cell surface protein and transcript levels. Levels of circulating immune complex (CIC) were measured in plasma by spectrophotometer. C3 was measured in serum by nephelometry in both controls and patients. C3d levels were estimated using ELISA Kit according to the manufacturer’s instructions. Clinical details of the patients were obtained from hospital records and DAS28 score was calculated accordingly. The methodologies employed were semi-quantitative RT-PCR, ELISA, Flow cytometry, nephelometry and spectrophotometry to meet the objectives of the study.

7.4 Standardization of Methods

Protocols for semi-quantitative RT-PCR, Flow cytometry and circulating immune complex were extensively standardized.

7.5 Highlights of the Results

**Expression of CR1 in controls and patients**

- The results showed a drastic decline in the levels of surface expression of CR1 on leucocytes in patients as compared to the controls. The decline (according to the mean value of MFI) in CR1 expression was in the order of neutrophils > lymphocytes > monocytes. The levels of CR1 in the cell surface and in transcript correlated positively both in patients and controls.

- The levels of CR1 transcript declined significantly in both PBMCs and neutrophils in patients as compared to the controls.
Summary and Conclusions

- Neutrophils expressed the highest levels of CR1 in controls and were the cells in which CR1 got maximally down-regulated in patients.

These observations suggested that a reduced level of CR1 on the cell surface and transcript level contributes to the drastic decline of CR1 on the leucocytes in patients as compared to controls.

**Correlations of CR1 transcript and its relationship with the disease activity**

- Levels of CR1 PBMC transcript correlated negatively with CIC in both controls and patients and also there was a significant positive correlation observed between CR1 PBMC transcripts with C3 controls. There was a significant positive correlation observed between CR1 PBMC and neutrophil transcripts with C3d levels in both controls and patients.

This suggested an intimate link of CR1 with the disease pathology.

- No correlation of the level of neutrophil CR1 transcript with both CIC and C3.

- Our study shows no significant correlation between CR1 PBMC transcript and DAS28 score in 0 months but there was a significant positive correlation in 3 months of follow up studies.

- Levels of CR1 neutrophil transcript correlated positively with DAS28 score in 6 months of follow up study.

Thus, a close relationship between the levels of CR1 transcript and the disease activity was envisaged.

**Expression of CR2 in controls and patients**

- There was a drastic decline in the levels of surface expression of CR2 on leucocytes in patients as compared to the controls. The decline (according to the mean value of MFI) in CR2 expression was in the order of neutrophils > lymphocytes > monocytes. The levels of CR2 on the cell surface and the level of transcript correlated positively both in patients and controls.

- The levels of CR2 transcript declined significantly in both PBMCs and neutrophils in patients as compared to the controls.
Neutrophils expressed the highest levels of CR2 in controls and were the cells in which CR2 got maximally down-regulated in patients.

These observations suggested that a reduced level of CR2 on the cell surface and transcript level contributes to the drastic decline of CR2 on the leucocytes in patients as compared to controls.

**Correlations of CR2 transcript and its relationship with the disease activity**

- Levels of CR2 PBMC transcript correlated positively with the disease activity parameters like CIC in controls only.

  This suggested an intimate link of CR1 and CR2 with the disease pathology and the balance between these two proteins were abrogated and disease association with RA.

- Levels of CR2 PBMC and neutrophil transcript correlated positively with C3 in patients.

- Levels of CR2 PBMC and neutrophil transcript correlated positively with C3d in patients, however, no correlation with the controls.

  This may indicate the reactive increase in production of these proteins rather than their consumption.

- Our study shows no significant correlation between CR2 PBMC transcript and DAS28 score in 0 months.

- Levels of CR2 neutrophil transcript correlated positively with DAS28 score in 3 and 6 months of follow up study.

  Thus, a close relationship between the levels of CR1 transcript and the disease activity was envisaged.

**Correlations of clinical parameters (CIC, C3 and C3d) with disease activity**

- Levels of CIC were significantly higher in patients as compared to controls.

- Significant positive correlation between CIC and DAS28 score in both 3 and 6 months of follow up studies.
Summary and Conclusions

- Levels of C3 were significantly higher in patients as compared to controls.
- There was significant positive correlation between C3 and DAS28 score in 6 months of follow up study.
- Levels of C3d were significantly higher in patients as compared to controls.
- There was significant positive correlation between C3d and DAS28 score in both 3 and 6 months of follow up study.

To conclude, leucocyte CR1 and CR2 declined markedly in patients with RA. Levels of CR1 correlated negatively with the activity and severity of the disease. To the best of our knowledge, this investigation has been the first of its kind. The investigation was initiated in view of the distinct lacunae in understanding of the role of CR1 and CR2 and its modulation in RA. The thesis suggests a) an intimate link of reduced levels of CR1 and CR2 with the pathophysiology of RA, b) a composite mechanism for lower levels of CR1 and CR2 in this disease and c) the factors involved in the regulation and modulation of CR1 and CR2 expression in this context. Since the levels of CR1 and CR2 showed disease related changes, further studies on the status and role of these proteins might help in the development of effective therapeutics for this disease.
7.6 Interrelationships of CR1, CR2, C3, C3d and CIC with the pathophysiology of Rheumatoid Arthritis (RA)

- Normal function of CR1 is IC clearance, B cell regulation and control of complement activation.
- Normal function of CR2 is B cell activation and to a small extent clearance of IC uptake by Leucocytes.
- In RA, reduced levels of CR1 and CR2 transcript may lead to B and T cell dysregulation. This may contribute to the formation of auto antibodies.
- Autoantibodies form Immune complexes, which don't get cleared efficiently because of low CR1 levels. This causes CIC overload, further manifested because of low CR1, since CR1 controls complement activation. Low levels of CR1 and CR2 expression in patients may, thus, contribute significantly to the pathological manifestation of RA.
- This is further confirmed by follow up of patients and correlation of CR1 and CR2 with C3, CIC, C3d and DAS 28.
- Cause-effect relationships of the upregulation of CR1 and CR2 on improvement of health condition along with treatment need to be investigated in future.
- A detailed follow up study is planned for future to get further confirmation on the relationships of CR1 and CR2 with the pathophysiology of RA and their importance as putative disease markers.

Note: The above chart is drawn on the basis of our findings (white font colour) and background information (black font colour).
8. BIBLIOGRAPHY


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APPENDIX

Materials

Chemicals and biochemical used for the experimental work are listed below.

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<thead>
<tr>
<th>Chemical</th>
<th>Source</th>
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<td>Absolute ethanol</td>
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Appendix

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<td>Xylene cyanol</td>
<td>Sigma Chemical Co. (USA)</td>
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</table>

Buffers

1.5% Agarose: 1.5 gm agarose in 100 ml of 1X TE buffer, 0.5μg/ml of ethidium bromide was added to the melted agarose.

Electrophoresis buffer for DNA (TAE buffer, 50X): 242 gm Tris-base, 18.6gm ETDA and 57.1 ml glacial acetic acid was dissolved in 800ml of DDW. pH was adjusted to 8.0 and volume was made upto 1L with DDW.

75% Ethyl Alcohol: 75% of absolute ethyl alcohol was mixed with 25ml of DDW.

10X MOPS running buffer: 16.7gm of MOPS (0.4 M, pH 7.0), 2.72 gm sodium acetate (0.1 M) and 0.74 gm ETDA (0.01 M). The buffer is adjusted to pH 7.0 with 1M NaOH and sterilised by autoclaving.

10X PB (0.01 M): 296.5 mg sodium hydrogen phosphate (NaH₂PO₄·2H₂O) and 1.44 gm di sodium hydrogen phosphate (NaH₂PO₄·2H₂O) was dissolved in 60ml of DDW and the pH was adjusted to 7.4 and volume made to 100ml.
**1X Phosphate Buffer Saline (PBS, pH 7.4):** 9 gm of NaCl was dissolved in 100 ml of 10X Phosphate Buffer (PB). The volume made to 1L with DDW.

**Polyethylene glycol (PEG 6000, 22%):** Prepared by dissolving 22gm of PEG 6000 in 100ml of Phosphate Buffer Saline (PBS, pH 7.4).

**Red Cell Lysis buffer (RCL buffer):** 8.26gm ammonium chloride (NH₄Cl, 154Mm), 1.0gm of Potassium bicarbonate (KHCO₃, 10mM) and 0.036 gm Ethylene diamine tetraacetate EDTA (0.1 mM) were taken and volume made to 1liter with DDW.

**RNA denaturing buffer:**

<table>
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<td>Deionized formamide</td>
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<tr>
<td>3.5ml</td>
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<td>formaldehyde</td>
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<tr>
<td>1.5ml</td>
<td>10x</td>
<td>MOPS buffer</td>
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ANNEXURE

Published Paper


Published Abstracts

1. Rozaleen Dash, Nitin Dhochak, Uma Kumar, Ashok Kumar and Nibhriti Das. Role of Complement Receptors CR1(CD35), CR2(CD21), CR3(CD11b/CD18) and CR4 (CD11c/CD18) in Rheumatoid Arthritis. 35th Indian Immunology Society conference, December 12-14, 2008, Bhubaneswar, Orissa, India. Pg no.56.

2. Rozaleen Dash, Uma Kumar and Nibhriti Das. Expressions of leucocyte complement receptors CR1 (CD35) and CR2 (CD21) in Rheumatoid Arthritis. 37th Indian Immunology Society conference, February 07-09, 2011, Srinagar, India. Pg no. 105.

3. Rozaleen Dash, Uma Kumar and Nibhriti Das. Levels of complement receptors CR1 (CD35) and CR2 (CD21) in the blood cells in relations to Rheumatoid Arthritis. 5th congress of the federation of Immunological Societies of Asia Oceania (FIMSA), March 14-17, 2012, New Delhi, India. Pg no. 131.

4. Rozaleen Dash, Uma Kumar and Nibhriti Das. Expression and significance of complement receptors (CR1 and CR2) in Rheumatoid Arthritis. 39th Indian Immunology Society Conference, November 09-11, 2012, Banaras Hindu University, Varanasi, India. Pg no. 45.


6. Rozaleen Dash, Uma Kumar, Maumita Kanjilal, Basanti Biswal and Nibhriti Das. Interrelationships of CR1, CR2, C3, C3d and C1q with the pathophysiology of Rheumatoid Arthritis. Abstract published at 40th Indian Immunology Society Conference, November 15-17, 2013, University College of Medical Sciences (Delhi University), New Delhi, India. Pg no.66.
Presentations in conferences

1. **Rozaleen Dash**, Nitin Dhochak, Uma Kumar, Ashok Kumar and Nibhriti Das. "Role of Complement Receptors CR1 (CD35), CR2 (CD21), CR3 (CD11b/CD18) and CR4 (CD11c/CD18) in Rheumatoid Arthritis" presented at 35th Annual Conference of Indian Immunology Society (December 12-14, 2008) held at Bhubaneswar, Orissa, India.

2. Participated in Rheumatology Update – 2009 (22nd February, 2009) organized by Delhi Rheumatology Association and Department of Medicine, AIIMS, New Delhi, India.

3. Participated in International Seminar on Complement in Health and Disease (An Indo-Dutch collaboration) (February 27th, 2010) held at All India Institute of Medical Sciences, New Delhi, India.

4. **Rozaleen Dash**, Uma Kumar and Nibhriti Das. “Expression of leucocyte complement receptors CR1 (CD35) and CR2 (CD21) in Rheumatoid Arthritis” presented at 37th Annual Conference of Indian Immunology Society (February 07-09, 2011) held at Sher-I-Kashmir Institute of Medical Sciences, Srinagar, India.

5. Participated in International Conference on Plant Science in Post Genomic Era (ICPSPGE-2011) (February 17-19, 2011) held at School of Life Sciences, Sambalpur University, Jyoti Vihar, Orissa, India.

6. **Rozaleen Dash**, Uma Kumar and Nibhriti Das. “Levels of complement receptors CR1 (CD35) and CR2 (CD21) in the blood cells in relations to Rheumatoid Arthritis” presented at 5th Congress of the Federation of Immunological Societies of Asia Oceania (FIMSA) (March 14-17, 2012) held at New Delhi, India.

7. Participated in one day National Symposium on New Horizon in Basic and Clinical Research (April 16th, 2012) organized by Society of Young Scientists, All India Institute of Medical Sciences, New Delhi, India.

8. **Rozaleen Dash**, Uma Kumar and Nibhriti Das. “Expression and significance of complement receptors (CR1 and CR2) in Rheumatoid Arthritis” presented at 39th Annual Conference of Indian Immunology Society (November 09-11, 2012) held at Institute of Medical Sciences, Banaras Hindu University, Varanasi, India.

9. Participated in Immunology day celebration (April 29th, 2013) organized by Indian Immunology Society, All India Institute of Medical Sciences, New Delhi, India.

10. **Rozaleen Dash**, Uma Kumar, Maumita Kanjilal, Basanti Biswal and Nibhriti Das. “Interrelationships of CR1, CR2, C3, C3d and CIC with the pathophysiology of Rheumatoid Arthritis” presented at 40th Indian Immunology Society Conference (November 15-17, 2013) held at University College of Medical Sciences (Delhi University), New Delhi, India.