Chapter 1

General introduction
"The third thing which should be noted in the podex is the double sac which in its lower proportion projects towards the pubic bone and appears visible to the observer as soon as the uterus, already mentioned, presents itself to view" (page 147 of De Formatione Ovi et Pulli, 1621\(^1\)). This was the first description by Hieronymus Fabricius ab Aquapente, a student and successor of the famous Andreas Vesalius (1514-1564), of a bursa near the terminal gut in chickens, which was later named after him: the bursa of Fabricius\(^2\). Nearly four centuries later, it was Bruce Glick at Ohio State University who functionally investigated the bursa of Fabricius by removing it from 3 weeks old young chicken embryos at the time when the bursa grew most rapidly\(^3\). By serendipity, his graduate student, Timothy Chang, needed birds to develop antibodies against Salmonella and injected 6-months old chickens with Salmonella-type O antigen not knowing they were bursectomized\(^2\). Unexpectedly, several of the injected chickens died and those that survived did not produce antibodies. Intrigued rather than disappointed by this event, Glick ended up demonstrating that the absence of the bursa of Fabricius was responsible for the failure of these chickens to produce normal antibody titers\(^4\). These studies on Bursa-derived cells (B-cells), according to several worthwhile of a Nobel prize\(^5\), were followed by studies by Miller et al. discovering the role of thymus-derived cells (T-cells) in immunity\(^6\). Together these research lines have formed the foundation of current immunological concepts involving the cellular and humoral immunity. However, with respect to autoimmune diseases, including rheumatoid arthritis (RA), the pathologic mechanisms underlying the autoinflammatory deregulation of both the cellular and humoral immune system are still being unraveled.

This thesis focuses on the role of the humoral immune system in RA patients in two ways: first, by investigating clinical and immunological effects of an anti-CD20 monoclonal antibody (Mabthera®, Rituxan®, rituximab) that specifically targets B-cells in RA patients and second, by studying the clinical and immunological effects of high dose chemotherapy (HDC) followed by autologous haematopoietic stem cell transplantation (HSCT) which non-specifically targets proliferating immune cells, including B- and T-cells. In this chapter an introduction will be given on rheumatoid arthritis, the humoral immune system and the rationale of specific and non-specific targeting of the humoral immune system. Chapter 2 focuses on the management of RA patients and the expanding role of B-cell depleting therapy in the rheumatological practice.
Rheumatoid arthritis

RA is a chronic systemic inflammatory disease, mainly characterized by symmetrical synovitis of diarthrodial joints resulting in pain, stiffness and loss of function. RA has a wide clinical spectrum varying from mild symptoms to severe inflammation and joint damage. Additionally, a wide variety of extraarticular manifestations may develop, e.g. rheumatoid nodules, vasculitis, lymphadenopathy, serositis and amyloidosis. The worldwide prevalence of RA is estimated to be 0.5-1% of the total population. RA affects women more often than men. RA patients have a significantly increased risk of death, which can be explained by several factors, e.g. chronic exposure to inflammation and cumulative toxicity of immunosuppressive drugs, resulting in an increased risk of infectious, hematologic, cardiovascular, gastrointestinal and respiratory disease. In addition, several risk factors have been identified that contribute to the development or severity of RA including genetic factors (HLA ‘shared epitope’, PTPN22 and Traf-C5 locus), estrogens (protective effects of pregnancy and oral contraceptives), smoking, coffee consumption and formal education.

The diagnosis of RA is based upon a composite of clinical and laboratory observations. However, criteria are needed for both epidemiological studies and clinical trials. Therefore, the classification criteria of the American College of Rheumatology were developed and commonly used to identify a category of ‘typical’ RA patients (Table 1). It is important to realize that the ACR criteria have a sensitivity of 77-95% and specificity of 85-95% to diagnose RA, when set out against the ‘gold standard’ of physician diagnosis. The immunological processes involved in the inflammation of synovium in the joints of RA patients are depicted in figure 1. Macroscopically, inflamed joints of RA patients typically show pannus formation with neovascularization, villous hyperplasia of the synovial membrane and fibrin deposits. Microscopically, infiltration of activated macrophages, T-cells, B-cells and plasma cells can be observed including signs of extinguished chronic inflammation by fibrosis of the synovial tissue. In addition, several studies have reported the formation of germinal center-like structures with follicular dendritic cells. Of note, circulating autoantibodies can be detected years before clinical onset of disease, supporting the notion that autoimmunity precedes clinically overt autoimmune disease. Due to the long subclinical phase of the disease, little is known about
Table 1 At least 4 of the following criteria, defined by the American College of Rheumatology, have to be met for the classification as rheumatoid arthritis (RA)\textsuperscript{18}.

<table>
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<th>Criteria</th>
<th>Condition</th>
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<td>Morning stiffness of &gt;1 hour most mornings for at least 6 weeks</td>
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<td>Arthritis and soft-tissue swelling of &gt;3, present for at least 6 weeks</td>
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<tr>
<td>Arthritis of hand joints, present for at least 6 weeks</td>
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<tr>
<td>Symmetric arthritis, present for at least 6 weeks</td>
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<td>Subcutaneous nodules in specific places</td>
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<td>Rheumatoid factor at a level above the 95th percentile</td>
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<td>Radiological changes suggestive of joint erosion</td>
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Figure 1 B-cells have a central role in the humoral immune system and the possible contribution of B-cells in RA-related synovial inflammation are depicted, from left to right; a) noncognate help for T-cell activation; b) efficient antigen-presentation, especially for recall antigens; c) antibody production; d) cytokine production that support survival of other mononuclear cells; e) production of chemotactic factors responsible for leucocyte migration and development of granulation tissue (i.e. pannus).
the events that trigger the abnormal immune response in RA. With respect to established RA, it is generally accepted that macrophage activity is involved in cartilage and bone destruction, radiographically visible as erosions and joint space narrowing. Recently, however, studies have showed an increased plasma cell infiltration at the junction sites of bone and synovium and increased osteoclastic activity in RA patients.

Taken together, it remains challenging to unravel the exact immunological pathophysiology of RA. Several important issues remain unsolved, such as the individual contribution of each infiltrating immune cell population in RA synovium, the origin of infiltrating cells and the role of RA-specific autoantibodies.

The humoral immune system in RA

As mentioned above in a historical perspective, the adaptive immune system is divided into a humoral, B-cell centered system and cellular, T-cell centered system. The core of humoral immune reaction is represented by the interaction of specific antibodies with foreign antigens. Antibodies are present in blood and other bodily fluids, hence the annotation ‘humoral’.

Thus, B-cells have a central role in the humoral immune system and the major physiologic functions of B-cells are: a) precursors of antibody producing plasma cells; b) noncognate help for T-cell activation; c) efficient antigen-presentation, especially for recall antigens; d) cytokine production that support survival of other mononuclear cells; e) production of chemotactic factors responsible for leucocyte migration and development of granulation tissue; and f) sustaining immunological memory. Many alterations in the physiologic processes of B-cell immunology have been described in RA patients, as discussed below (Figure 1).

In the synovium of RA patients, B-cells are found in close proximity of T-cells and even germinal center-like structures have been described. Studies of animal models have convincingly shown that B-cells are essential for T-cell activation and that other antigen-presenting cells were unable to substitute for the maintenance of T-cell activation. In addition, RA synovium was shown to express increased levels of CXCL-12 (i.e. stromal cell derived factor or SDF-1), which was shown to contribute to the resistance of B-cells to apoptosis. Moreover, plasma cells were found to migrate towards gradients of SDF-1 and
in its turn SDF-1, together with IL-5, IL-6, TNF-α and CD44 ligand, prolonged the longevity of plasma cells. Altogether these data showed that B-cells not only play an essential role in the inflammation of RA synovium, but also that synovial accumulation of B-cells and plasma cells is determined by the dysregulated expression of cytokines, costimulatory molecules and B-cell survival factors in RA.

Autoreactive B-cells are antecedents of plasma cell-derived production of autoantibodies. Currently, rheumatoid factor (RF) and anti-cyclic citrullinated protein antibodies (ACPA) are the most specific for RA, respectively 80% and 95%\(^3\). Both autoantibodies can be detected before overt clinical disease has manifested and high concentrations are found in synovial fluid of RA patients\(^3\). It is still matter of debate whether these autoantibodies cause disease symptoms in RA or merely are a bystander effect\(^3\). However, transfusion of RF autoantibodies into healthy persons did not result in arthritic disease\(^3\). Nevertheless, the presence of autoantibodies is enough to increase and perpetuate inflammation in RA synovium. It has been demonstrated, for example, that autoreactive B-cells possess a specialised ability to bind any antigen that is already part of an antibody immune complex, through which they can present a variety of antigens to antigen-specific T-cells and thus activate them\(^3\). Additionally, autoantibody formation can activate the complement system and bind to, and activate, macrophages in the synovium. As a result, immune-complex activated macrophages produce pro-inflammatory cytokines that perpetuate inflammation and induce joint destruction. Collectively, it can be concluded that B-cells have several potential pathologic functions in RA pathology, which include 1) the initiation of autoimmunity by uptake and presentation of autoantigens, 2) the maintenance of autoimmune disease by promoting T-cell activation and chemoattracting other mononuclear cells, and 3) the perpetuation of autoimmune disease by autoantibody production and providing autoreactive memory.

**Targeting the humoral immune system in RA**

The importance of the humoral immune system in rheumatoid arthritis (RA) has long been subject of debate and, despite the early discovery of autoantibodies, the focus of the majority of investigations was on T-cell mediated processes due to the genetic association of HLA genes and the development of RA. However,
B-cells and the humoral immune system regained interest with the successful introduction of B-cell targeted therapies in RA patients\textsuperscript{33}, as extensively described in chapter 2 of this thesis. In contrast to specifically targeting B-cells, high dose chemotherapy (HDC) is a strong anti-proliferative and immunosuppressive treatment depleting actively proliferating immune cells, including B-cells and T-cells\textsuperscript{36}. In order to better cope with the strong immunosuppressive effects of HDC it is followed by autologous transplantation of hematopoietic stem cells (HSCT), in order to shorten the leucopenic period in which patients are at high risk for infections\textsuperscript{37}. The rationale of HDC+HSCT is based upon the complete eradication of auto-reactive lymphocytes, including B-cells producing pathogenic autoantibodies. Previous studies have shown the feasibility, safety and efficacy of HDC+HSCT in refractory RA patients\textsuperscript{38,39}. In addition, it was observed that a good response to HDC+HSCT was associated with significant reduction in synovial T-cell infiltration\textsuperscript{40}. However, thus far, the effects of HDC+HSCT on the humoral immune system have not been reported yet.

**Outline of this thesis**

The aim of this thesis was to unravel the role of the humoral immune system in RA patients by employing new immunosuppressive strategies, i.e. specific B-cell depletion with rituximab and non-specific lymphoablative treatment with HDC+HSCT. This thesis evaluates the clinical benefit of these strategies as well as the immunological changes that coincide with clinical improvement. By combining clinical outcome with immunological parameters of the humoral immune system, these studies provide a unique approach to investigate pathologic mechanisms in RA.

**Chapter 2** reviews the therapeutic management of RA patients and the expanding role of B-cell depletion in the rheumatologic practice. **Chapter 3** describes the depleting effects of rituximab in peripheral blood, bone marrow and synovium. **Chapter 4** demonstrates the association between CD79a+ plasma cells in synovium of rituximab treated RA patients with clinical disease activity and B-cell reconstitution. In **chapter 5** lymphocyte distribution in blood, bone marrow and synovium of RA patients is compared to healthy persons, indicating a skewed distribution of B-cells in RA patients. In **chapter 6** we conducted an
open-label, two-center study to compare the clinical efficacy and safety of two B-cell depleting treatment strategies in refractory RA patients. Chapter 7 describes the important methodological bias of epitope masking in patients treated with rituximab. In chapter 8 the sensitivity and specificity of different markers for B-lineage cells is discussed. Chapter 9 studies the survival of human plasma cells in the experimental setting of organ cultures. In chapter 10 the efficacy of HDC+HSCT from a patient-centered perspective is studied. Chapter 11 investigates the immunological effects of HDC+HSCT on the normal and autoreactive humoral immune system of autoantibody-positive RA patients. Chapter 12 is dedicated to a hypothesis-driven review on the pathogenicity of RA-specific, anti-cyclic citrullinated autoantibodies (ACPAs) and the potential role of autoreactive plasma cells in RA.
References


