Targeted therapies in rheumatoid arthritis
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CHAPTER 3

Response to rituximab in patients with rheumatoid arthritis in different compartments of the immune system

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In the last decade, with the implementation of new targeted drug therapy, the efficacy of the treatment of rheumatoid arthritis (RA) has increased enormously. However, a subset of patients still does not respond to any of the available treatments. At this moment, the chimerical anti-CD20 monoclonal antibody rituximab is one of the therapeutic options in RA patients who failed anti-TNFα therapy. After infusion it leads to a fast and almost complete depletion of CD20 positive B cells in the peripheral blood. In contrast to the peripheral blood compartment that has been studied quite extensively, only limited data exist on the effect of rituximab on other compartments of the immune system, like lymph nodes, bone marrow and synovial tissue. By studying the mode of action in the different inflammatory compartments, we may gain a more detailed understanding of the pathogenesis of RA and the mechanism of action of rituximab. In light of personalized medicine, this knowledge could lead to the identification of patients with a potentially improved clinical response, compared to for example patients who might need intensified dosing regimens or may better be treated with other drugs.

ROLES OF B CELLS IN THE IMMUNE SYSTEM

It is well established that B cells are responsible for antibody production. More recently, studies on B cell function have implicated multiple additional roles for B cells in the immune system. B cells leave the bone marrow as immature B cells and complete maturation into mature naïve B cells in the peripheral lymphoid tissues. Once activated, B cells secrete polarized arrays of cytokines, dependent on the mode in which they are stimulated. They can also activate T cells and thereby stimulate their proliferation, differentiation and polarization, and enhance/sustain the activation of primed T cells. Furthermore, B cells cross-talk with dendritic cells in the process of T cell activation, but the precise mechanisms have not yet been elucidated. B cells may also acquire a regulatory phenotype and, by secretion of IL-10, can suppress both Th1 and Th2 polarization, and inhibit antigen presentation and pro-inflammatory cytokine production by macrophages. Finally, B cells belong to the cells that may regulate lymphoid tissue architecture and ectopic lymphoid neogenesis.

After an infection has been successfully terminated, most B cells and short-lived plasma cells undergo apoptosis, but some survive, due to competition for survival niches in lymphoid tissue, as memory B cells or as long-lived plasma cell and can survive for years. They are able to respond quickly following a second exposure to the same antigen.

Taken together, a body of data indicates that B cells, besides being precursors of plasma cells, play an active role in the formation of the immunological environment and immunological memory, and, similar to T cells, have the capacity to regulate the extent, direction and quality of inflammatory responses.

ROLE OF B CELLS IN RA: HOW COULD RITUXIMAB INTERFERE?

RA is a chronic inflammatory disease characterized by synovial tissue inflammation in the joints. Both clinically and biologically, RA is a heterogeneous condition that is probably driven by different immune mechanisms in different patient subgroups. Currently, few biomarkers are known that can be used to classify these different patient groups. The most important are the presence of bone erosions on conventional X-rays and the presence of the auto-antibodies
IgM-rheumatoid factor (RF) and anti-citrullinated peptide antibodies (ACPA) in the peripheral blood. These auto-antibodies are present very early in the disease course, even before synovial inflammation occurs. Data from experimental studies, showing the stimulating effects of ACPA or immune complexes containing rheumatoid factor or ACPA on tumor necrosis factor production by macrophages, provide a link between B lineage cells on the one hand and the clinical signs and symptoms of RA on the other, in light of the known correlation between synovial TNF expression and scores for arthritis activity.

The clinical benefit of rituximab treatment further supports the notion that B cells play a key role in the pathogenesis of RA, at least in the two-thirds of the patients responding to rituximab. An important biomarker predictive of response is the presence of autoantibodies, i.e., seropositivity for IgM-RF and/or ACPA at baseline. The clinical responses are consistently enriched in patients who are IgM-RF and/or ACPA positive compared to those who are autoantibody negative. Interestingly, even within the autoantibody positive subgroup rituximab induces a heterogeneous decrease in disease activity with ACR20, 50 and 70 responses of 51%, 27% and 12%, respectively. This clearly shows that positivity for IgM-RF and/or ACPA (measured by the anti-cyclic citrullinated peptide test) only partly explains the response to rituximab. The level of total IgG has also been shown to be an independent factor predictive of response to rituximab in RA patients, suggesting the presence of other, as yet unknown auto-antibodies.

The presence of lymphocyte aggregates in the inflamed synovium containing B cells that are often surrounded by large numbers of plasma cells, also suggests an important role for B cells in RA. The expression of the costimulatory molecule CD86 is increased on RA B cells. In line with this observation, T cell activation in synovial lymphocyte aggregates has been shown to be dependent on the presence of B cells. Furthermore, B cells belong to the cells that regulate lymphoid tissue architecture and may also regulate ectopic lymphoid neogenesis. In this way, rituximab could probably also induce a decrease in disease activity in RA by indirect effects on cells other than CD20+ B cells. Finally, in addition to B cells, there is also a small population of CD20+ T cells in RA patients. Depletion of these CD20+ T cells may be an additional mode of action of rituximab. It is currently unknown to what extent rituximab interferes with the different roles of B cells.
PRECLINICAL DATA ON THE MODE OF ACTION OF RITUXIMAB

Rituximab is an antibody directed against the 33-37 kDa, non-glycosylated phosphoprotein CD20 that is expressed on the surface of almost all B cells, except for stem cells, pro-B cells and plasma cells 31. Knowledge about the biology of CD20 is limited, partly because it has no known natural ligand and CD20 knockout mice show normal B cell development and function 32. Some insight into its function has come from work showing that CD20 is resident in lipid raft domains of the plasma membrane where it probably functions as a store-operated calcium channel following ligation of the B cell receptor for antigen 33. Recent data suggest that CD20 is important for T cell independent antibody responses 34. CD20 is an attractive target for monoclonal antibodies for several reasons: First, it is expressed at high levels on most B cells, allowing dense accumulation of the monoclonal antibodies (mAbs) on the cell membrane. Second, it does not become internalized or shed from the plasma membrane following mAb treatment. This allows mAbs to persist on the cell surface for extended periods and permit a sustained immunological attack from complement (complement-dependent cytotoxicity) and FcR-expressing innate effectors (antibody-dependent cellular cytotoxicity). Third, it is a transmembrane molecule with a short extracellular domain, therefore the mAb is close to the target cell, which may be important in recruiting complement. Finally, CD20 can generate

![Figure 2: Potential roles of B cells in controlling macrophage numbers in the synovium. B cells can differentiate into auto-antibody producing plasma cells. The auto-antibodies can stimulate Fc-receptors that are abundantly expressed on macrophages, thereby providing survival signals to the macrophages 17. Second, auto-antibody-containing immune complexes can activate complement, causing release of C5a, which is a macrophage chemottractant 74-75. Third, B cells can produce cytokines and activate T cells to produce for instance TNFα and GM-CSF. Pro-inflammatory cytokines can subsequently stimulate stromal cells to further increase GM-CSF production. Both TNFα and GM-CSF are important in macrophage recruitment, survival, and retention 69. IL-1 = interleukin-1](image-url)
transmembrane signals when engaged by a mAb by triggering cell cycle arrest and sometimes programmed cell death (reviewed in 35). There is in vitro evidence that both complement-dependent and antibody-dependent cytotoxicity are involved in the mechanism of action of rituximab; these two mechanisms are additive in a number of models 36-38. The direct inhibitory effect on the cell cycle is less defined and the data on this subject are quite controversial 35.

Animal studies have suggested that rituximab-induced B cell depletion varies among different tissues and that different effector mechanisms may be important for B cell depletion in the different compartments. Mice treated with anti-CD20 monoclonal antibodies showed depletion of B cells from lymph nodes within days, whereas it took weeks to see this effect in the peritoneal cavity. In addition, in a human CD20 transgenic mouse model, marginal-zone B cells of the spleen and B cells located in the germinal centres of lymphoid tissues appeared to be partly resistant to depletion 39,40. In this mouse model, complement-dependent cytotoxicity seemed to play a dominant role in B cell depletion in the splenic marginal zone B cell compartment, whereas Fc receptor mediated mechanisms (like antibody-dependent cytotoxicity) were most important in the elimination of circulating B cells as well as lymph node and splenic follicular B cells 39. As part of the preclinical toxicology evaluation during development of anti-CD20 therapy, cynomolgus monkeys were treated with a fully humanised monoclonal antibody against CD20. It was shown that B cells in the peripheral blood compartment were fully depleted and also fully recovered both after the first treatment and after retreatment. B cells in the tissues were depleted to a lesser extent than those in the peripheral blood, with a more pronounced depletion in the spleen than in the lymph nodes. Of note, there was large variability in depletion between animals 41.

Some insight into how B cell depletion may result in amelioration of auto-immune disease comes from work in the murine collagen-induced arthritis (CIA) model in RA. B cell depletion inhibited antigen-specific CD4-positive T cell expansion in one study 4. In another study, it was effective in the prevention of CIA, but B cell depletion after collagen immunization did not have a significant effect on arthritis progression or severity. This may suggest that B cell depletion will be most effective early in the development of arthritis and that sustained B cell depletion is required to inhibit the evolution of joint inflammation and destruction 42.

A major conclusion of the preclinical studies is that B cells that reside in lymphoid tissue seem to be partly resistant to depletion by anti-CD20 therapy, either by defective effector mechanisms or because these B cells have a specific phenotype, and that sustained B cell depletion seems to be required to inhibit the evolution of arthritis.

B CELL DEPLETION IN PERIPHERAL BLOOD AND BONE MARROW OF RA PATIENTS

Rituximab induces a transient, nearly complete depletion of CD20 positive B cells in peripheral blood within a few hours 43. Only B cell subsets from the immature phase in the bone marrow up to the memory B cells stage are directly depleted, since stem cells, pro-B cells and plasma cells do not express CD20 35; however, indirect effects on other cells may occur. Small numbers of B cells may persist after rituximab treatment in a proportion of patients. These B cells are mainly CD20 negative plasma cell precursors 44,45. The level of depletion in the peripheral blood is probably not related to the serum levels of rituximab, so other explanations for
persistence of both CD20 positive and –negative B lineage cells in the peripheral blood need to be investigated 46.

The mean time frame of B cell return is 8 months, but this period is highly variable between patients 41. In some patients B cells are depleted for years although this was not analyzed with high-throughput FACS-analysis 47. The B cell repopulation in the peripheral blood shows a specific pattern; first, immature B cells reappear, followed by naïve B cells. Memory B cells show a slow and delayed repopulation and the level of these cells can stay reduced for more than 2 years 43,47-49. Interestingly, early clinical relapse after rituximab treatment has been associated with a higher proportion of memory B cells before treatment and with a higher percentage of memory B cells in the repopulating cells 43,48,49. Furthermore, a higher number of CD20 negative preplasma cells before treatment with rituximab has been associated with incomplete B cell depletion and worse clinical response 45.

Other biomarkers of response recently identified include the presence of a type I interferon signature in peripheral blood mononuclear cells at baseline that was negatively correlated with the response to rituximab in 3 independent cohorts 50. In addition, a single study suggested that BlyS promoter polymorphism may be associated with the response to rituximab therapy 51. Peripheral blood levels of BlyS, a B cell maturation factor, increase immediately after rituximab induced B-cell depletion 52,53. This may contribute to the regeneration of B cell subsets.

After rituximab treatment, there is a gradual decrease in IgM-RF and ACPA levels, which is more pronounced than the reduction of total antibody concentrations and serum levels of antibodies against microbial antigens, such as *Streptococcus pneumoniae* and *Clostridium tetani* 54. Similarly, serum levels of free light chains, which are products of short-lived plasma cells with a half life of 2 to 3 days, decrease after treatment. In contrast to ACPA and RF, free light chains only decrease in clinical responders to treatment. These changes in free light chains are not found after infliximab treatment, which suggests a rituximab-specific indirect effect on CD20 negative short-lived plasma cells in responders 55. Of interest, there was no effect of rituximab on the IgA-expressing plasma cell subset with a mucosal origin 56. These cells may continuously circulate in the peripheral blood after rituximab treatment, suggesting resistance to depletion of a mucosal B cell subset. Future research should address the question as to whether the presence of these cells can explain non-response in a subset of the patients.

In contrast to the marked B cell depletion observed in the peripheral blood of nearly all RA patients, relatively high numbers of persistent CD20 positive B cells may be found in the bone marrow after rituximab treatment 57-60. Recently, it was shown that the remaining B cells are mostly memory B cells, while a pronounced depletion of naïve B cells was observed. Of note, compared to patients who were treated with rituximab for the first time, the bone marrow of retreated patients contained a significantly lower proportion of memory B cells, whereas the number of immature B cells was increased 59. This finding suggests that the renewal of memory B cells is impaired and corresponds to the delayed repopulation of memory B cells observed in the peripheral blood. Furthermore, patients who have a high proportion of mature (naïve plus memory) B cells in their bone marrow before rituximab therapy, show a clinical response of a relatively short duration that is accompanied by an early return of B cells in the peripheral blood: there may have been incomplete depletion of the mature B cells in these patients 57. In line with this notion, it was shown that the clinical response to rituximab was preceded by a decrease in memory B cells in the bone marrow 58. These data are also consistent with peripheral
blood analysis and suggest that disease progression after rituximab treatment is probably due to survival of memory B cells. It should be noted however that data on the effects of rituximab on the bone marrow were achieved in small cohorts of patients with different co-medications and remain to be replicated in larger independent cohorts.

There are at present no studies on the effects of rituximab on the spleen or lymph nodes of RA patients. Splenic CD20 positive B cells were completely depleted in patients with non-Hodgkin’s lymphoma after rituximab treatment in combination with chemotherapy; in the lymph nodes there was only partial depletion.

**THE EFFECTS OF RITUXIMAB ON THE SYNOVIAL CELL INFILTRATE IN RA PATIENTS**

In the inflamed synovial tissue of RA patients, there are various forms of lymphocyte infiltration. In about half of the RA patients CD20 positive B cells are present in the synovium, mostly found in lymphocyte aggregates together with T cells and surrounding fields of plasma cells (Figure 1). Studies on the depletion of synovial tissue B cells by rituximab consistently show that this depletion is variable. This variability cannot be explained by differences in rituximab serum levels. Possibly, different synovial expression levels of complement inhibitory factors or B cell survival factors are responsible for this variability in B cell depletion, although so far no clear cut relationship has been found with the expression of the survival factors CXCL12, APRIL (A Proliferating Inducing Ligand) and BlyS (B Lymphocyte Stimulator). Although the decrease of synovial tissue B cells after rituximab varies between patients, this decrease is statistically significant on the group level as early as 4 weeks after initiation of therapy. Of interest, the degree of depletion of synovial B cells is not related to the clinical response.

As mentioned, B cells have different roles in the immune response; thus rituximab could induce indirect effects on immune cells other than B cells. An interesting effect of rituximab treatment is seen on plasma cells: the change in synovial plasma cells is predictive of the clinical response after 24 weeks. No statistically significant reduction of synovial plasma cells was found on the group level, which could be explained by a highly variable response: in some patients there is a marked decrease in plasma cells, whereas in others plasma cell numbers in the synovium are unaltered. This decrease was correlated with the decrease of serum levels of ACPA at week 16. Consistent with these findings, serum levels of free light chains only decrease in clinical responders.

Rituximab treatment is associated with a decrease in synovial T cells and disrupts the presence of synovial aggregates and follicular dendritic cells. Rituximab treatment also resulted in a significant decrease in the number of synovial macrophages. It is at present not entirely clear what the underlying mechanisms are. Potential mechanisms on how B cells could influence macrophage numbers in the inflamed synovium are shown in Figure 2. The effect of rituximab on macrophage numbers confirm and extend the notion that these cells are key players in common final pathways involved in RA pathogenesis; treatments with a completely different mechanism of action may ultimately affect macrophage numbers in the synovium. The consistent relationship between the change in CD68 positive macrophages and clinical improvement after antirheumatic therapy was recently also confirmed for rituximab (Figure 3).
In summary, the data on the effects of rituximab on the synovial tissue show that this treatment affects B lineage cells with an indirect effect on other cells, like T cells and macrophages; only depletion of plasma cells correlates with clinical response. Future treatment strategies could include combination therapy of rituximab with therapies that target plasma cell survival and differentiation in non-responders to rituximab alone. Whether this approach leads to improved efficacy with acceptable safety remains to be proven.

**CONCLUSION**

Putting everything together, what would be the key mechanism that determines clinical response? First of all, different studies showed that CD20 positive B cells may be incompletely depleted in bone marrow and synovial tissue, while lymphoid tissue has not yet been extensively studied. Variability in CD20 positive B cell depletion may be explained by pharmacogenetic factors, such as Fcγ receptor polymorphisms, individual differences in survival factors such as BLyS or by differences in RA immunopathology. Some clinical non-responders may have a B cell subtype involved in their disease that is less sensitive to rituximab. In line with these hypotheses, a number of studies identified patient-specific factors that might predict clinical response: the autoantibody profile, the serum level of IgG, the presence of a type I interferon signature in peripheral blood mononuclear cells and the number of pre-plasma cells in peripheral blood and memory B cells in peripheral blood and bone marrow.

**Figure 3.** Correlation between the change in the disease activity score in 28 joints (DAS28) and the change in synovial sublining CD68 expression, when comparing trials of different antirheumatic drug treatments (R = 0.91, P = 0.0002). Anti-MCP-1 = anti-monocyte chemotactic protein-1; DMARD = disease-modifying antirheumatic drug; MTX = methotrexate.
Apart from differences in CD20 positive B cell depletion, clinical response may be determined by indirect effects on other inflammatory cells, such as plasma cells, T cells and macrophages. As shown, the size of the decrease in (pre-)plasma cells and free light chains predicts clinical response. Furthermore, it remains to be shown whether clinical response is associated with a decrease in a specific T cell subset (Table 1). Future work may provide a better understanding of the role of B cells and plasma cells in different tissues in the pathogenesis of RA. It can also be anticipated that (combinations of) biomarkers will provide an increasingly important tool to further optimize the cost-effectiveness of rituximab in the treatment of RA.

Table 1. Mechanism of response to rituximab

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<td>memory B cells</td>
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<td>BlyS polymorphism*</td>
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* IFN=interferon, BlyS = B Lymphocyte Stimulator

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