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Understanding response to rituximab treatment in rheumatoid arthritis through immune fingerprinting of T and B cells

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Chapter 1

General introduction

THE HUMAN IMMUNE SYSTEM

Our immune system protects us from the billions of different pathogens which may attack us during our lifetime. There are several layers of defense which our immune system adopts in the fight against the invaders[1]. Anatomical barriers, such as the skin and mucosal tissue, represent the first layer of defense which aim to physically block the entrance of the pathogen. When this fails, the second layer of defense comes into play, which is the innate immune system. The cells and components of the innate immune systems act quickly in response to a breach in the anatomical barriers and they adopt a series of generalized strategies to get rid of the invader which relies on shared commonalities between different kind of pathogens. This includes the release of antimicrobial proteins, the engulfment (phagocytosis) of the pathogens and the release of inflammatory cytokines to recruit other immune cells to the site of infection. These non-specific mechanisms, which might initially help in the clearance of the pathogen, may also damage the surrounding (uninfected) tissue and do not provide a long-standing protection upon re-infection with the same pathogen. On top of that during evolution more specific and durable strategies developed to fight invading pathogens, i.e., adaptive immune responses[2]. Cells of the adaptive immune system, i.e., the T and B cells, are equipped with a unique cellular receptor, the T-cell receptor (TCR) and the B-cell receptor (BCR) respectively. These receptors can specifically recognize parts of an invading pathogen, termed antigens, ensuring a higher level of specificity and efficacy in the clearance of the pathogen. Although the adaptive immune response takes longer to mount, the adaptive immune cells can persist much longer in our body even after the pathogen has been eliminated, providing a lifelong memory of each pathogenic encounter and protection for subsequent infection[3].

THE ATTACK OF THE CLONES: ADAPTIVE IMMUNE RESPONSES

An adaptive immune response begins when a naive T or B cell, i.e., a newly generated and "immature" T or B cell, recognizes a (pathogenic) antigen via its TCR or BCR (Figure 1). The binding of the antigen to the receptor (antigen recognition) triggers the cell to undergo several rounds of proliferation, a process called *clonal expansion*. This process ensures the formation of an "army" of T or B cell clones all equipped with a TCR or BCR able to recognize the invading pathogen. Additional cues in the form of cytokines steer the acquisition of extra effector functions or *differentiation*: B cells will differentiate into plasma cells, which secrete their BCR in the form of an antibody; T cells will differentiate into cytotoxic T cell (CD8+ lineage), able to mediate the killing of other (infected) cells, or into T helper cells (CD4+ lineage) which will provide additional help and coordination to both innate and adaptive immune cells. For example, follicular T helper cells provide help to cognate B cells (i.e., B cells specific for the same antigen) for the generation of high affinity antibodies. This happens through the formation of a germinal center,

a microanatomical structure which transiently forms in secondary lymphoid organs, where clonally expanding B cells modify their BCR with the introduction of affinity increasing point mutations, a process called somatic-hypermutation (SHM) and/or by switching their functional domain, a process called class-switch recombination (CSR)[4]. The resulting secreted high affinity antibodies are generated later during the course of an adaptive immune response but are extremely efficient in the binding and clearance of the pathogen. Once the pathogen has been completely eliminated from the body, clonal contraction occurs, i.e. clonal T and B cells are removed from the body and only few cells remain, the memory T and B cells which will persist in our body providing lifelong protection against the pathogen. In fact, upon a second encounter with the same pathogen, these memory T and B cells will re-activate and mount a much faster and more efficient immune response, which will result in the rapid elimination of the pathogen.

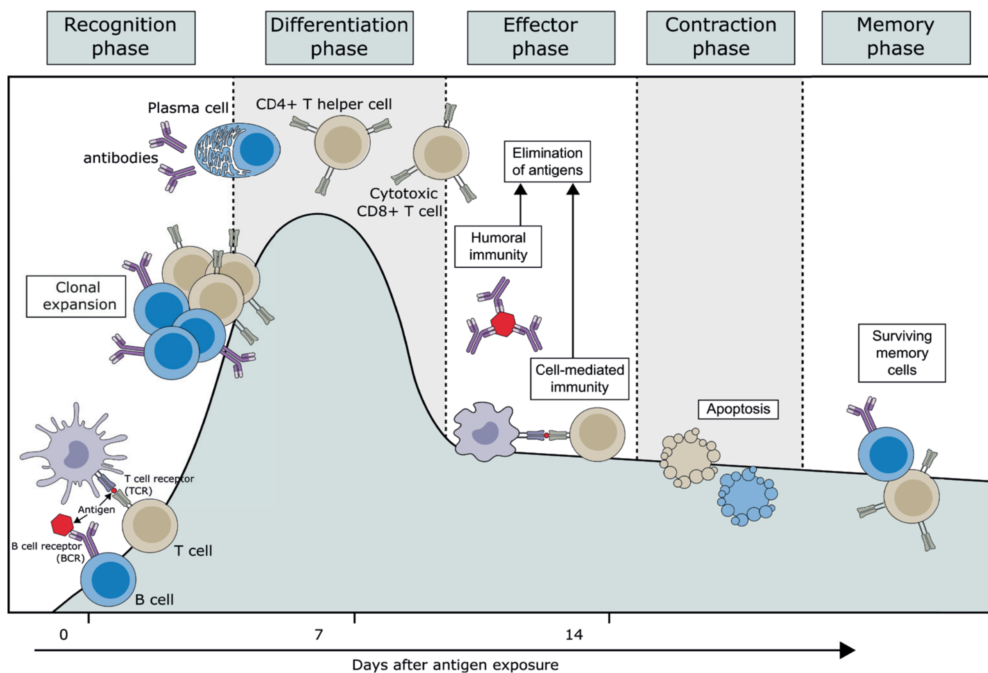

Figure 1| Adaptive immune responses. Schematic representation of the different phases during an adaptive immune response. In the recognition phase, naïve T and B cells recognize parts of the pathogen (= antigen) via their T or B cell receptor and consequently undergo several rounds of proliferation or clonal expansion. In the activation phase, T and B cells differentiate into effector cells such as antibody producing plasma cells, CD4+ T helper cells or CD8+ cytotoxic T cells which mediate the elimination of the pathogen (effector phase). In the contraction phase after the complete clearance of the pathogen, the clonally expanded T and B cells undergo cell death via apoptosis and only few cells remain, i.e., the memory cells which will provide life-long protection against the encountered pathogen (memory phase).

Courtesy of Niels J.M.C. Verstegen. Adapted from Abbas AK, Lichtman AH, Pillai S. Basic immunology: functions and disorders of the immune system, 4th edition, Philadelphia, 2012, Saunders. *©2014, with permission from Saunders, an imprint of Elsevier Inc.*

T AND B CELLS TURINING TO THE DARK SIDE: AUTOIMMUNITY

Apart for its specificity and memory formation, another key feature of the adaptive immune system is its ability to distinguish self from non-self. During their development in the primary lymphoid organs (thymus for T cell and or bone marrow for B cell), T and B cells are selected: those that carry a TCR or BCR able to bind autoantigens, i.e. antigens expressed by our body's own cells and tissues, are physically or functionally suppressed. This mechanism, known as central tolerance, ensures that autoreactive T and B cells are removed from the repertoire prior to being released in the circulation. Since this process is not 100% effective, autoreactive T and B cells which escape central tolerance can also be removed while circulating throughout our body, a process termed peripheral tolerance. Despite this double layer of control, some autoreactive T or B cells manage to circulate freely throughout our body and - once activated after encounter with their cognate autoantigen - will mount an immune response against it. These autoimmune responses develop in the same way other adaptive immune response develop, leading to the formation of effector autoreactive T cells and effector autoantibody secreting B cells. These cells will attack healthy cells and tissues within our body, thus leading to inflammation and tissue damage, typical features of autoimmune diseases. Additionally, given that the antigen eliciting an autoimmune response is always present within the body, another feature of autoimmune diseases is their chronicity.

RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is a chronic autoimmune disease which affects 1% of the population worldwide. It is characterized by inflammation of the synovium in peripheral joints which, if untreated, can lead to tissue damage and cartilage and bone destruction. The exact pathophysiology of this disease is still not completely understood, but genetic predisposition, together with non-genetic and environmental risk factors are thought to slowly and continuously contribute to the clinical onset of this disease[5]. RA is nowadays seen as a multiphasic disease, with different molecular and cellular mechanism being involved at each stage and the need for subsequent "hits" to push the disease to its next stage (Figure 2). Yet, the exact pathological immune pathways involved into the establishment of clinically defined RA can vary on the individual levels. In fact, a recent study analyzing the cellular composition in the site of active disease, i.e. the synovium, revealed the presence of six different "pathotypes" based on the composition of the immune cell infiltrate, which also correlated with differences in treatment response[6].

Of interest in this context, seropositive RA patients, i.e. patients with detectable autoantibodies, show a larger influx of B cells in the synovium and a more aggressive disease course[7]. Two types of these autoantibodies are included in the diagnostic criteria for RA: rheumatoid factor (RF) targeting the functional domain (i.e. the Fc tail) of IgG antibodies and the anti-citrullinated protein antibodies or ACPAs, targeting the post-

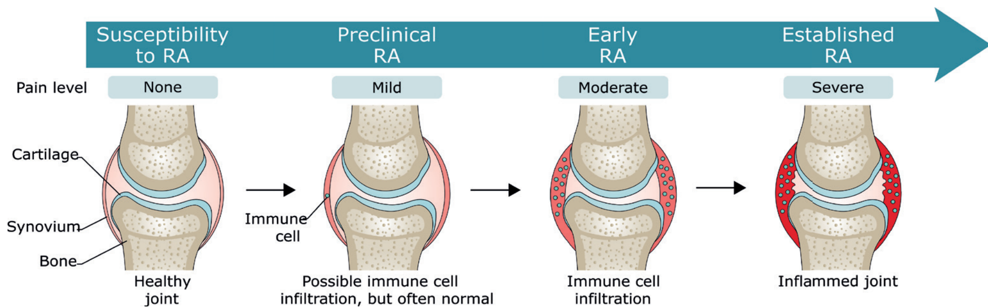

Figure 2| Rheumatoid arthritis is a multi-stage disease. Schematic representation of the different developmental stages of rheumatoid arthritis. Healthy individuals with genetic and non-genetic risk factors for RA development present with a healthy synovium and no evidence of autoimmune responses ("RA susceptibility phase"). In the next disease stage ("Preclinical phase") signs of (auto)immune activation are detected in the circulation while the synovium still looks healthy or with minor immune cell infiltration. In the "Early RA" phase, the immune cell infiltration in the synovium becomes detectable and individuals start to experience complains in their joints (subclinical synovitis) but criteria for RA classification are not yet met. Further progression of the disease finally leads to the clinically relevant and classifiable RA ("Established RA").

Adapted from Smolen, J., Aletaha, D., Barton, A. et al. Rheumatoid arthritis. Nat Rev Dis Primers 4, 18001 (2018), *©2018, with permission from Macmillan Publishers Limited, part of Springer Nature.*

translational protein modification citrullination. And more types of autoantibodies have been recently identified in sera of RA patients[8]. Such autoantibodies can be detected years before the onset of clinical symptoms, indicating that autoimmune responses driven by autoreactive B cells have been initiated long before the onset of clinically definable disease[9]. Additionally, several studies have shown that autoantibody levels increase in blood just prior to disease onset while at the same time the specificity of antigen binding diversifies, a process termed epitope spreading[10,11]. However, once the disease is set, the dynamics of autoantibodies seems to stabilize thus indicating that generation of new autoreactive B cells is important in the initiation of the pathological condition but not in its perpetuation.

RITUXIMAB IN THE TREATMENT OF RA

A renewed interest in the role of B cells in the pathogenesis of RA arose also with the introduction of biologicals, i.e. monoclonal antibodies targeting a specific immune cellular or molecular component, in the management of the disease. From a research perspective, the use of such targeted therapies offers the opportunity to study the effect of the temporary depletion of a cellular or molecular component on the disease activity. This was the case for rituximab, a chimeric monoclonal antibody directed against the B cell surface molecule CD20. A single treatment with rituximab induces > 98% depletion of the B cells in peripheral blood lasting for at least 4-5 months and clinical response is achieved in 50-65% of treated patients[12,13]. Despite the clear efficacy of B cell depletion in the treatment of RA, response on the individual level may vary and is difficult to

predict. This might be due to the above-mentioned variable contribution of the B cell compartment in disease pathogenesis. In fact, patients seropositive for both RF and ACPAs benefit the most from B cell depleting therapy with rituximab[14]. Additionally, timing and depth of B cell depletion has also being implicated in treatment failure even when a very clear correlation has never been demonstrated[15].
Another factor which might influence response to rituximab and biologicals in general is the development of immune responses directed against the biological agent, i.e. immunogenicity. Such responses may lead to the formation of anti-drug antibodies or ADAs, which can increase the drug clearance due to the formation of ADA-drug complexes[16]. Consequently, patients that develop ADAs may show reduced drug levels in the serum, which can ultimately lead to reduced treatment efficacy[17,18].

IMMUNE FINGERPRINTING OF T AND B CELLS: TCR AND BCR REPERTOIRE ANALYSIS

As mentioned before, any adaptive immune response, be it physiological or pathological, starts with the binding of an (auto)antigen to a TCR or BCR. This may trigger the clonal expansion of the T or B cell that carries the respective TCR or BCR. Overall, this leads to an increase in the amount of TCRs/BCRs triggered in this clonal expansion. Thus, identifying and quantifying all the different TCRs and BCRs present within a person at a given time, i.e. monitoring the repertoire in this individual, could give us information about the ongoing (adaptive) immune responses.
However, this analysis is complex and complicated by the extreme diversity of TCR and BCR repertoires which has been estimated to be at least 10^{15} for TCRs and 10^{12} for BCRs[19,20]. This diversity serves to virtually recognize every pathogen which may attack us during our lifetime. To generate such diversity specific mechanism are used. TCR and BCR transcripts are not individually encoded within the DNA but rather are assembled by combining alternative gene segments during the development of each single T or B cell. In this process of “VDJ recombination” variable (V), diversity (D) and joining (J) segments are cut-and-pasted together to generate a functional receptor RNA transcript. As a result, every single T or B cell in our body carries a unique TCR or BCR, which can be therefore thought of as a molecular “fingerprint” of each T or B cell. Advances in next-generation sequencing (NGS) technologies allow researchers to identify and quantify thousands and thousands of single TCRs and BCRs at once. This is achieved using of *ad-hoc* methodologies for the amplification and sequencing of TCR and BCR RNA molecules, which due to their nature can’t be amplified with routine sequencing procedures[21,22]. In fact, In the work described in this thesis these methodologies have been used to monitor the immune system.

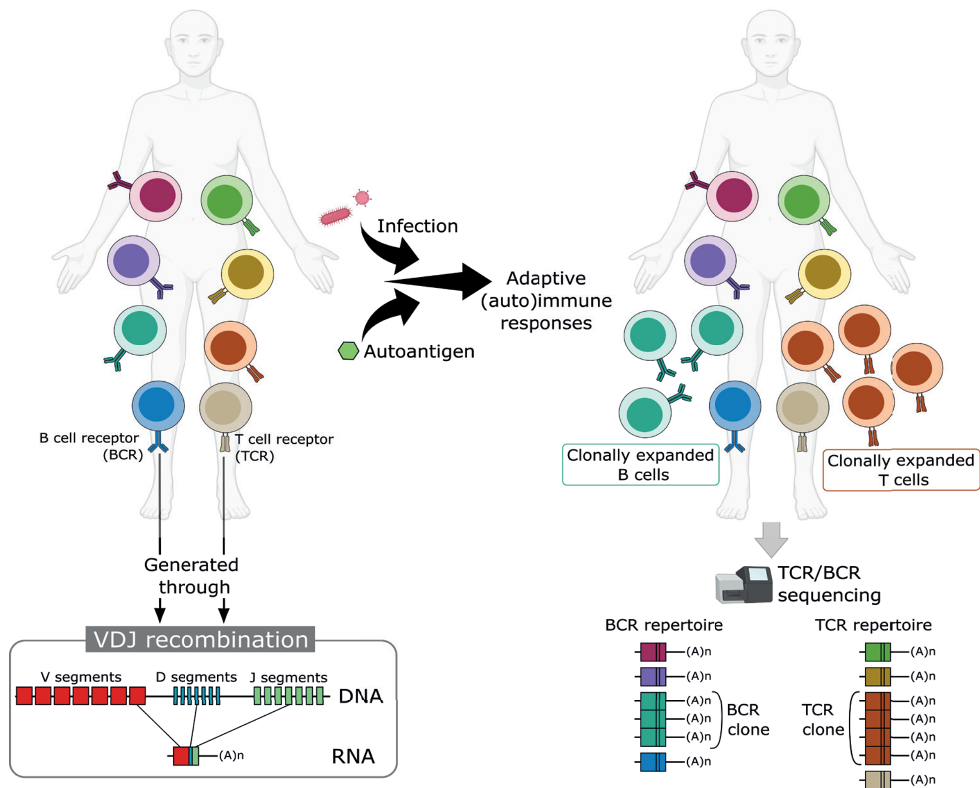

Figure 3| TCR and BCR repertoire as fingerprint of adaptive immune responses. T-cell receptors and B-cell receptors are uniquely generated in each T and B cell during development via genomic recombination of different gene segments (V = variable; D = diversity; J = joining), a process termed "VDJ recombination". When adaptive immune responses are initiated, antigen specific T and B cell undergo clonal expansion and consequently also the TCR and BCR of clonally expanded T and B cells will be overrepresented compared to TCR and BCR of not-clonally expanded T and B cells. By sequencing the TCR or BCR molecules within an individual, i.e. the TCR or BCR repertoire, we can evaluate and monitor adaptive immune responses going on in that individual.

THESIS OUTLINE

The aim of the work described in this thesis is to get more insight into B-cell depletion therapy in the treatment of RA, to understand the cellular mechanisms behind its efficacy and its failure, with the final purpose to improve treatment strategies towards a more successful clinical response. To this end, we analyzed the BCR and TCR repertoire in RA patients starting treatment with the B cell depleting agent rituximab at different time points after treatment and in different tissues. Additionally, we evaluated the development of immunogenic responses against rituximab and specifically the involvement of T cells in such responses.

In **part I** of this thesis we evaluate the effect of B-cell depleting therapy on the B-cell

receptor (BCR) repertoire in RA patients starting on rituximab. In **chapter two** we applied RNA-based next-generation sequencing (NGS) of the BCR repertoire in paired peripheral blood and synovial tissue samples collected from 24 seropositive RA patients treated with rituximab. Clonal expansion, mutation load and clonal overlap was assessed before treatment and at week 4 and at week 16 or 24 after treatment and correlated to the patients' clinical response. In **chapter three** we analyzed another prospectively followed cohort of 31 RA patients starting on rituximab and developed a new UMI-based NGS pipeline to analyze the BCR repertoire in peripheral blood samples obtained at one, three, six and twelve months after treatment. By monitoring the percentage of unmutated BCRs in the repertoire as a proxy for the percentage of naïve B cells in peripheral blood we defined in each patient the timepoint at which depletion and repopulation were achieved. We analyzed the correlation of early or late B cell depletion or repopulation with a patient's clinical response and studied the development of anti-rituximab antibodies.
In **part II**, we shifted the focus to T cells and their involvement in the development of immunogenic responses against rituximab. In **chapter four** we developed a new methodology to select antigen responsive T cell clones by combining TCR repertoire sequencing with *in vitro* stimulation assays. In particular, we analyzed the dynamics of antigen-induced clonal expansion after *in vitro* stimulation and applied established bioinformatic routines for transcriptomic expression analysis to identify the over-expanded antigen-responsive clones. In **chapter five** we applied this novel methodology to identify and characterize rituximab responsive T cell clonal responses in RA patients undergoing rituximab treatment. We investigated the cytokine profile of such responses in patients which did or did not develop anti-rituximab antibodies. In the latter patients, *in vitro* identified rituximab responsive TCR clones were traced back in *ex vivo* peripheral blood TCR repertoires obtained at different timepoint after rituximab treatment.
Finally, in **chapter six** the findings and results presented in this thesis are summarized and discussed in the light of current literature and with an outlook to future perspectives..

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