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

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Chapter 6

General discussion

GENERAL DISCUSSION

Despite the great research advances in dissecting the mechanisms underlying rheumatoid arthritis onset and development, the exact pathophysiology of this disease remains unsolved. In the past years, the introduction of biologicals, and in particular of therapeutic monoclonal antibodies (mAb), has constituted a major breakthrough in the clinical management of the disease. Although these new drugs have proven effective, in some patients, remission or low disease activity is only partially or temporally achieved. Understanding the mechanism behind this incomplete response might lead to improved therapies and in ultimately to improved quality of life for patients. This thesis describes our efforts to elucidate the mechanisms behind response to B-cell depletion therapy using rituximab in rheumatoid arthritis. We evaluated how both direct and indirect effects can influence clinical response to the treatment. In this chapter, we present a summary of the obtained results and discuss the implications in the context of current literature, with an eye on prospective research challenges and additional unmet needs.

PART I: BCR repertoire analysis for the evaluation of clinical response to rituximab

In the first part of this thesis, we focus on evaluating the direct effect of rituximab treatment on the B cell compartment by monitoring the B cell receptor (BCR) repertoire and correlating changes in the repertoire with clinical response. In **chapter two** we analyzed the BCR repertoire in paired peripheral blood and synovial tissue samples obtained before and at 4 and 16/24 weeks after rituximab treatment. We observed significant changes in the peripheral blood BCR clonality at 4 weeks post-treatment: There were fewer BCR clones, but also more dominant clones with increased mutation rate. The latter was also observed in synovial tissues but only at the later follow-up timepoint. At week 4 post-treatment, non-responders showed a higher clonal overlap with the baseline repertoire, indicating that clinical response might be linked to the extent of disruption of the (pre-treatment) repertoire. In **chapter three** we confirmed and further extended these results in a new cohort of RA patients starting rituximab therapy. The BCR repertoire was analyzed in peripheral blood samples obtained before and at one, three, six and twelve months after treatment. The significant changes observed in the peripheral blood BCR repertoire at one month post treatment, i.e. more expanded, less diverse and more mutated, started to revert at six month post-treatment indicating a re-establishment of the pre-treatment situation. We speculated that the percentage of unmutated BCR clonotypes in the peripheral blood repertoire could be used as a proxy to monitor the fraction of naïve B cells. Using this parameter, we defined a depletion and a repopulation timepoint in each patient and correlated this to clinical response. We observed that timing of depletion and repopulation did not predict response to treatment at six or twelve months. However, repopulation within the first six months

did significantly correlate with decreased disease activity in the subsequent period (i.e. 6-12 months post-treatment).
The finding described in chapter two and three that the post-rituximab repertoire consists of fewer, more expanded and more mutated BCR clonotypes is not surprising. This residual B cell population is most likely composed of plasmablasts and plasma cells which do not express CD20, the molecular target of rituximab. Therefore, these cells are not affected by this B cell depleting therapy. In other studies, phenotypical characterization of the post-depletion B cell compartment using highly sensitive flow cytometry showed the presence of a residual memory B cell population which was also the predominant re-populating fraction in patients with early relapse after B cell recovery[1–4]. Even though our RNA-based analysis is dominated by the BCR signal coming from plasmablasts/ plasma cells due to their higher RNA content (data not shown from our lab), we can't exclude that some of the residual dominant BCR clones observed in our analysis still represent memory B cells.
Yet, while the lack of CD20 might explain resistance to treatment, other explanations may be of interest. An alternative explanation could be that they are not reached by the treatment because they are homing into tissue where they are protected from cell death. This data acquired in chapter two seem to support this hypothesis: The synovial BCR repertoire did not showed changes in the number of (dominant) clones after rituximab treatment. Additionally, rather than a delayed direct effect of rituximab in the tissue, the increase in highly mutated BCR clones observed only a later timepoint could be a consequence of a reduced influx of (unmutated) naive B-cells from the peripheral blood, which is a mechanism thought to be important not only in the perpetuation but also in the onset of the clinical symptoms of the disease[5–7].
The synovium is not only the target tissue in RA. In fact, *in situ* B cell proliferation, affinity maturation, and autoantibody production has been observed in the synovium of RA patients within organized T- and B-cell follicle-like structures termed ectopic germinal centers[8–10]. Similar to what is observed in both primary and secondary lymphoid organs, these anatomical structures provide a protective niche for B cells, which ensures cellular survival, also in the absence of typically necessary cues, most importantly antigen stimulation[11,12]. This protective niche, in addition to the reduced penetrance of the drug within tissues might explain why B cells in the synovium are resistant to rituximab depletion. An alternative approach to target those B cells would be to first mobilize them into the circulation where rituximab is most effective. This approach, which has already shown promising results in mice, is currently being tested in SLE patients using belimumab, a monoclonal antibody directed against B-cell activating factor (BAFF), which increases circulating memory B cells most likely by inducing migration and/or retention of tissue resident B cells in the circulation[13–15].
An interesting point which could not be addressed in chapter two is the relation between

the peripheral blood and the synovial tissue BCR repertoire before and after rituximab treatment. In untreated RA patients, comparison between these anatomical locations has revealed that only few BCR clones found in the synovium could be traced back in the peripheral blood[16]. This result is in line with earlier findings since the synovial B cell response is most likely very localized in the ectopic germinal centers mentioned above. In fact, also the clonal overlap between different joints or different locations within the same joint has been showed to be much lower compared to the overlap of the T cell compartment[17].

Furthermore, if we consider that B cells present in peripheral blood are "on the road" from or towards different anatomical locations, we might observe a higher overlap between the peripheral blood and the tissue repertoire when these anatomical locations are sampled at different timepoints. In this line of thinking, it would be interesting to investigate whether the residual BCR clones observed in peripheral blood after rituximab induced B cell depletion are present in synovium at earlier or later timepoints. If true, this would indicate that disease perpetuating B cells are locally protected from depletion by rituximab in the synovium and/or other tissues via the mechanisms mentioned above but can still circulate to and from the tissues via the peripheral blood. Monitoring the overlap between the peripheral blood BCR repertoire at the time of B cell repopulation and the synovium at the time of disease relapse, might reveal that rituximab can only temporarily affect the pathological B cells by constraining their migration through the peripheral blood into to the synovium, the so-called "road-block hypothesis"[18].

Lastly, analysis of the overlap between peripheral blood and synovium repertoire could provide even more insight when performed at the pre-clinical stage. Such analysis might in fact identify the population of autoreactive B cells on its road to infiltrate the synovium and start the inflammatory process, which will ultimately lead to the development of clinical symptoms. Once the BCR sequences of these cells are identified, it could be used as a barcode to follow their evolution in time and perform deeper phenotypic analysis, in an approach we call "reverse phenotyping". This could ultimately lead to the discovery of additional molecular targets, that can be used to selectively treat these pathological B cells at the moment in which their contribution to the disease is thought to be at its highest[19].

An interesting result obtained from chapter three is the fact that patients which repopulated the B cell compartment within 6 months after treatment show an amelioration of disease activity in the subsequent 6 months. Disease improvement after post-rituximab B cell repopulation has been observed in other autoimmune disease[20]. This suggests that it is the rituximab induced B cell-repopulation, rather than the B cell-depletion itself, which has a beneficial effect on disease activity. It has been reported that repopulation of B cells after depletion starts with antigen naïve B cells[2,21]. Phenotypic analysis of repopulating B cells after rituximab treatment in patients with multiple sclerosis revealed a significantly

enrichment in transitional and mature naive phenotypes and a reduction in the frequency of memory B cells[22]. Thus, the mechanism behind the efficacy of rituximab treatment might lay in its ability to “reset” the (pathological) B cell compartment.
One of the new approaches to reset the B cell compartment is by using anti-CD19 chimeric antigen receptor (CAR) T cells. This treatment results in depletion of (CD19+) B cells, and induced an impressive 100% remission rate after 3 months of treatment in an experimental cohort of 5 SLE patients[23,24]. Analysis of the repopulating B cells again revealed a “reset” of the B cell compartment, with IgM B cells being the most abundant, while memory B cells were being observed in very low numbers. Compared to rituximab treatment, anti-CD19 CAR T cell therapy targets the entire B cell compartment and patients might stay immunocompromised for months. A possible alternative is the administration of CAR-encoding mRNA[25]. With this approach, the chimeric antigen receptor is only transiently expressed in T cells, ensuring reduced risk of off-target effects, and the possibility to fine-tune therapy intensity through repetitive administration.
Another more selective approach aiming for a B-cell reset which has reached clinical testing is the expression in T cells of a chimeric receptor containing the autoantigen recognized by B cells, i.e. chimeric autoantibody receptor (CAAR) T cells[26]. This approach, in which only autoreactive B cells are targeted, can only be applied in those autoimmune diseases where a pathogenic autoantigen is identified. This, unfortunately, is not the case in RA.
Yet it should be noted that these treatment approaches which aim to eliminate the autoreactive B cells, rather than interfere with the mechanisms which made them pathogenic, i.e. breach of tolerance, may not be curative: Once the autoreactive B cells against the given antigen are removed, new ones with new (pathogenic) specificities could still be generated. To circumvent this, a “complete reset” of the entire immune system might be needed and physiological tolerance re-established[27]. Indeed, autologous stem cell transplantation (ASCT) which resets the entire immune system has shown the highest drug-free remission success rate, However, the severe immunodeficiency induced by this therapy and the fact that both pathological and protective immunological memory is erased, makes this approach hardly applicable as a routine clinical practice.
Until the moment in which a drug-free remission therapy for the management of RA is achieved, future experimental treatment approaches should aim to prevent the establishment of clinical disease. In the pre-clinical phase, individuals “at-risk” already developed an autoimmune condition, i.e. they have circulating autoantibodies, but are not yet fulfilling the criteria for clinical disease, even though they may experience joint complaints. This phase, now referred to as the “window of opportunity” is raising more and more interest as a moment for therapeutic intervention by the rheumatologist. In the past years several trials performed on at-risk individuals or early-stage RA patients have been undertaken to investigate the safety and feasibility of preventive treatment

approaches using glucocorticoids, conventional disease modifying anti-rheumatic drugs (cDMARDs) or biologicals (reviewed in 28). While almost all treatment approaches showed increased beneficial effects on disease-related symptoms and inflammation, only the use of biologicals, in particular of rituximab or abatacept (which interferes with T cell activation), showed delayed or reduced risk to develop RA[3,29,30]. This confirms that targeting specific cellular or molecular components of the adaptive immune system, rather than a generalized action on the immune system, is the most effective strategy to interfere with disease progression. In this respect, given that it takes multiple "hits" to reach clinical onset of RA, additional research into the exact cellular and molecular mechanisms steering each subsequent "hit" are needed in order for clinicians to decide which treatment might optimally interfere with such mechanisms. Yet, the fact that not all "at-risk" individuals will eventually progress to fully established disease poses the ethical question to whether pre-clinical intervention on clinically "healthy" individuals is in fact feasible, also considering the psychological and physical side effects which such interventions might induce.

PART II: Developing TCR repertoire analysis to assess immunogenicity

In the second part of this thesis, we aimed to investigate an aspect which is known to influence clinical efficacy of rituximab and of other biologicals in general: drug immunogenicity. We aimed to use AIRR sequencing which, as mentioned before, delivers a *fingerprint* of ongoing immune responses within an individual, including anti-drug responses in treated patients. But first, we had to overcome a major limitation of this approach, namely the fact that no information about antigen specificity or (functional) phenotype of the receptor-carrying cells can be derived from the receptor sequence itself. In **chapter four** we developed a new methodology to identify antigen-specific immune responses by combining TCR repertoire sequencing with *in-vitro* antigen stimulation assays. We extensively analyzed the dynamics of antigen-induced clonal expansion after *in vitro* stimulation by performing TCR repertoire sequencing on *in vitro* PBMC cultures at different timepoints after stimulation. We applied bioinformatic methods routinely used in transcriptomic analysis to evaluate the changes in clonal frequency of individual TCR clones over time, and to select antigen-responsive clones as clones with a differentially increased frequency after antigen-stimulation. In addition, we showed how further phenotypical and functional characterization of the selected TCR clones can be easily associated by applying different *in vitro* culture settings (in particular addition of different cytokines) or performing additional cellular sorting (in particular sorting based on the activation markers CD154 and CD25). In **chapter five** we applied this methodology to evaluate immunogenic T cell responses against rituximab. PBMCs from rituximab-treated RA patients which developed anti-drug antibodies one year post-treatment were stimulated *in vitro* using a pool of rituximab-derived peptides. Rituximab-responsive TCR clones were identified in each patient as differentially over-expanded clones comparing the pre- and post-stimulation repertoire. Further characterization of the rituximab-responsive TCR clones revealed an intra- and inter-patient polyclonality, with no identical TCR clones, i.e. *public* clones, shared among different patients. Neither were other TCR proprieties shared, such as CDR3 length and charge, V-gene usage and V-J pairing. Finally, we traced back the *in vitro* identified rituximab-responsive TCR clones in peripheral blood samples obtained prior to and at one, three, six and twelve months post-treatment. Most of the rituximab-responsive TCR clones appeared in blood within the first month of treatment and persisted up to twelve months. In some cases, they could even be observed before treatment initiation.

The methodology presented in **chapter four** adds up to an increasingly growing list of methods and algorithms developed in the last years to derive information about receptor specificity from TCR repertoire data. In 2017 two major algorithms for epitope binding prediction were release, GLIPH and TCRdist[31,32]. These algorithms which rely on clustering based on sequence similarity, require a training dataset composed of known and validated TCR-epitope pairs, which impairs their application to study novel antigens. In this respect, our methodology offers the possibility to study the specificity against any kind of antigen, from a small antigenic peptide to a large complex molecule. Yet, the above-mentioned algorithms which improved even more by the recent implementation of machine learning approaches, offer the possibility to define the features determining the TCR-epitope binding which is a much more biological relevant information than just determining the TCR-epitope binding pairs. Another approach in which the application of machine learning provided interesting results is the use of total peripheral blood TCR repertoires to classify the immune status of an individual. In the first study published applying this approach in 2017, individuals were classified as being CMV positive or negative based on the presence of TCRβ sequences which were previously identified as being associated with CMV infection[33]. Two years later a similar approach was used to distinguish individuals affected by an autoimmune condition (rheumatoid arthritis and systemic lupus erythematosus) from healthy individuals[34]. Interestingly, even though the datasets used in both studies contained thousands of sequences, only a few hundred TCRs associated with the disease status were used to train the classifier. This confirms that specific adaptive immune responses are initiated by very few specific T- and B-cells. In order to improve its potential for experimental and clinical research even more, future studies involving AIRR sequencing should be performed on subsets of cells selected based on features of interest, for example antigen specificity or functional phenotype, and not on total T- and or B cells where the signal coming from the relevant specific adaptive immune responses is diluted and could be missed.

In the past years, research into immunogenic responses against biologicals has mostly focused on the assessment and characterization of its humoral manifestations, i.e. antidrugs antibodies (ADAs). This has revealed that the majority of ADAs are of the IgG subtype, with predominance of IgG4 in long-term re-treated patients, implying the presence of drug-specific CD4+ T helper cells which help cognate B cells to differentiate and class-switch into IgG-producing plasma cells[35]. Yet, analysis of T cell responses against a biological is scarcely performed in clinical research. In **chapter five** we present one of the first studies evaluating anti-drug T cell response against rituximab in treated individuals which showed that rituximab-responsive TCR clones can be found in the peripheral blood repertoire very early after treatment and precede the detection of anti-rituximab antibodies. Additionally, functional analysis revealed that rituximab-responsive T cells are able to produce IFN-γ, IL5 and IL10, the latter two being important for maturation and differentiation of antibody-producing B cells. One important missing point is whether such anti-drug responses can be detected also in treated patients which do not developed ADAs, and whether the responding T cells show the same functional phenotype as observed in ADA-developers. In other studies, drug-specific T cells derived from ADA positive patients have been shown to secrete a different cytokine profile compared to T cells from ADA negative patients, namely IFN-y, IL13 and IL10 in ADA positive and IL10 only in ADA negative[36,37]. It is well known that secretion of IL10 by T cells is an important mechanism for tolerance induction[38]. It can therefore be speculated that drug-specific T cells in ADA negative patients have a tolerogenic phenotype which prevents the development of anti-drug antibodies. Of interest in this context is a small study which showed that rituximab-specific T-regs could be induced through a desensitization protocol in a reactive patient with a consequent decrease in ADA levels[39]. Additional studies should focus on the identification and characterization of drug-specific T cells in both ADA positive and ADA negative patients to confirm the above-mentioned functional differences at a larger scale. This could in fact build a rationale to adopt tolerance inducing protocols aside to biologicals therapies in order to reduce immunogenicity.

Immunogenicity against biologicals is observed in about 1/3 of treated patients[40]. The generation of such an immune response against therapeutic antibodies can actually be considered a normal immune response against a novel protein, and therefore should be expected in all treated patients. In fact, the development of a more sensitive assays for the detection of anti-drug antibodies, namely the pH-shift anti-idiotype antigen-binding test (PIA) which allows the detection of both free and drug-complexed ADAs, revealed a much higher prevalence of anti-drug antibodies in treated patients than the commonly used and less sensitive antigen-binding test (ABT)[41]. Yet, patients which were defined as ADA positive using the PIA test showed the same sustained remission rate as patients defined as ADA negative, while none of the patients defined as ADA positive using the ABT test reached sustained remission[42]. This indicates that ADAs have clinical relevance only when they reach a concentration that influences drug availability in the serum.

This concept was very nicely described by *Schouwenburg PA et al*. and is reported in figure 1. When ADAs levels in serum are low (and can be detected only using PIA) the drug will be only partially neutralized, leaving enough free drug in the serum to ensure treatment efficacy. Once the level of ADAs increases (and can be detected also with ABT), more drugs will be neutralized, and therefore treatment efficacy starts to decrease with a reduction of clinical response. Another point which is clearly illustrated is the fact that at high drug concentrations, low ADA levels are observed. Thus, a possible solution to overcome immunogenicity-driven reduction on efficacy would be to maintain the level of the drug high enough to prevent a rise in ADA levels and/or ensure enough free drug in the serum. Adjusting the drug dosage depending on the availability of drug in the serum, which is the principle referred to as therapeutic drug monitoring (TDM), has already been proved to be effective in reducing treatment failure in the long-term compared to standard dosage[43,44]. This has led to the recent release of guidelines and consideration points for the application of TDM as proactive approach in clinical practice[45]. Such personalized approaches could ensure treatment efficacy even in the (inevitable) presence of ADAs with the additional beneficial effect of lowering treatment costs.

Concluding remarks and future perspective

Management of RA has undergone impressive advances in the last decades, and nowadays low disease activity or remission is an achievable goal. Yet, the studies presented in this thesis show how current treatment strategies are only partially effective and need improvements. In particular, the results presented demonstrate how prediction of clinical response to rituximab therapy remains challenging. This is probably due to the fact that both drug-related and patient-specific characteristics influence its efficacy. Some of these factors will be difficult to control, such as the distribution of the drug into the affected tissue. Others could be assessed prior to treatment initiation, such as

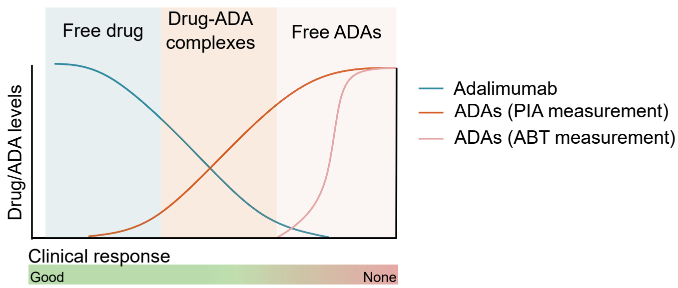

Figure 1 | Model for clinical relevance of ADAs. Schematic representation of the model explaining correlation between drug and anti-drug antibodies (ADAs) levels and clinical response.

Adapted from van Schouwenburg PA, Krieckaert CL, Rispens T, Aarden L, Wolbink GJ, Wouters D Long-term measurement of anti-adalimumab using pH-shift-anti-idiotype antigen binding test shows predictive value and transient antibody formation. Ann Rheum Dis 2013;72:1680 - 1686, *©2013, with permission from BMJ Publishing Group Ltd. & European League Against Rheumatism*

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the “pathotypes” with the best response rate, i.e., autoantibody positivity or adaptive immune cellular infiltration in the synovium. Yet, some factors only manifest once the treatment has been initiated, as for example the development of anti-drug antibodies.
The close monitoring of rituximab induced B cell depletion and most importantly of B cell repopulation after rituximab treatment as shown in **part I** might give valuable guidance for the decision to intensify or taper treatment in order to maintain clinical remission and/or low disease activity. In fact, since disease relapse mostly occurs after repopulation of the B cell compartment, detecting this event as early as possible might be of help for clinicians which could then monitor disease activity more closely and thus be able to intervene earlier at the first signs of disease re-awakening. Yet, several studies have now confirmed that B cell repopulation does not directly lead to disease relapse but could be the key mechanism behind induction of remission in rituximab treated patients. In this respect, future research into clinical efficacy of rituximab in RA patients should shift the timing of analysis from the rituximab-induced B cell depletion to the post-rituximab B cell repopulation, and to the moment of clinical relapse. In particular, phenotypic analysis and, most importantly, BCR repertoire analysis of the B cells at the two latter timepoints might reveal whether disease relapse is triggered by the re-appearance of pre-treatment B cells or by newly formed pathogenic ones. The first scenario would confirm that rituximab is not able to reach and/or affect pathogenic B cells; the second scenario would reveal that rituximab can affect the pathogenic B cells but not the mechanism behind their formation. In both cases, adjustment or change of the treatment strategy would be needed to ensure the patient’s clinical improvement.
The fact that current treatment in RA is not fully effective also reflects the lack of a fully defined mechanism explaining disease onset and perpetuation. In this respect the success (and failure) of therapeutic antibody therapies has helped in elucidating which immunological axes are a valuable target when aiming to reduce disease activity. Yet, whether such axis plays an important role also in disease onset still needs to be elucidated. More studies are needed which evaluate the effect of biological treatment on different cellular components of the immune system, and which study how such effects correlate with clinical response. Together with an improved understanding of how targeted treatments act, such studies could reveal which cellular mechanism are responsible for disease perpetuation. Additionally, future research in rheumatoid arthritis should shift its focus of investigation from the cellular and molecular mechanisms acting during established diseases to the cellular and molecular discrete events in the stepwise progression from asymptomatic to clinically relevant RA. The elucidation of the single consecutive “hits” needed to progress from one stage to the another could in fact help in the design of a series of targeting therapies which could subsequently be applied to delay the onset of clinical symptoms as much as possible.
Irrespective whether their molecular or cellular target is appropriate in relation to RA

pathophysiology, current biologicals treatments are impaired in their clinical efficacy by the development of immunogenic responses. These responses develop early after treatment initiation, as we showed in **part II**, and could be even present before treatment initiation, thus building a rationale for testing immunogenic responses in candidate patients before the start of therapy. The extensive research from the past years on the incidence of anti-drug antibodies in treated patients has led to the realization that such immunogenic response is inevitable. However, evaluation of their impact on treatment outcome revealed that not all immunogenic responses are clinically relevant. Therefore, future studies should investigate how such inevitable immunogenic responses could be steered to minimize their impact on clinical response for example by adjusting the dosage and/or interval of treatment or by adding concomitant immunosuppressant/ tolerance inducing agents.

Finally, both **part I** and **part II** demonstrate the potential of TCR and BCR repertoire analysis beyond providing a “sterile” catalogue of the ongoing (and past) adaptive immune responses within an individual. In chapter two and three we exploit the amount of somatic hypermutation found in the BCR sequence to derive the phenotype of the receptor-carrying B cell. In chapter three, we used the percentage of unmutated BCR clonotype in the repertoire as a proxy for the percentage of naïve B cell and monitored B cell depletion and repopulation after rituximab treatment in a much more sensitive way that conventional clinical routine could allow. In chapter four we combined TCR repertoire analysis with *in vitro* simulation assay and develop a methodology for the identification of antigen responsive TCR clones which could easily be adjusted to study any antigen of interest and easily scalable to large patients’ cohorts. These results show how the use of TCR and BCR repertoire analysis can be integrated in experimental and clinical practices. In the latter case, many studies have now shown the potential of repertoire data to classify disease status. Yet, in all these studies well-defined disease groups are compared and therefore disease-defining features might be enriched. Validation of such approaches in prospective studies may confirm whether such signatures are not only distinctive but also unique to a disease condition and could therefore be exploited for diagnostic purposes. In the future, complementation with more advanced artificial intelligence approaches could make it possible to define and predict the immune status of an individual by its TCR/BCR repertoire.

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