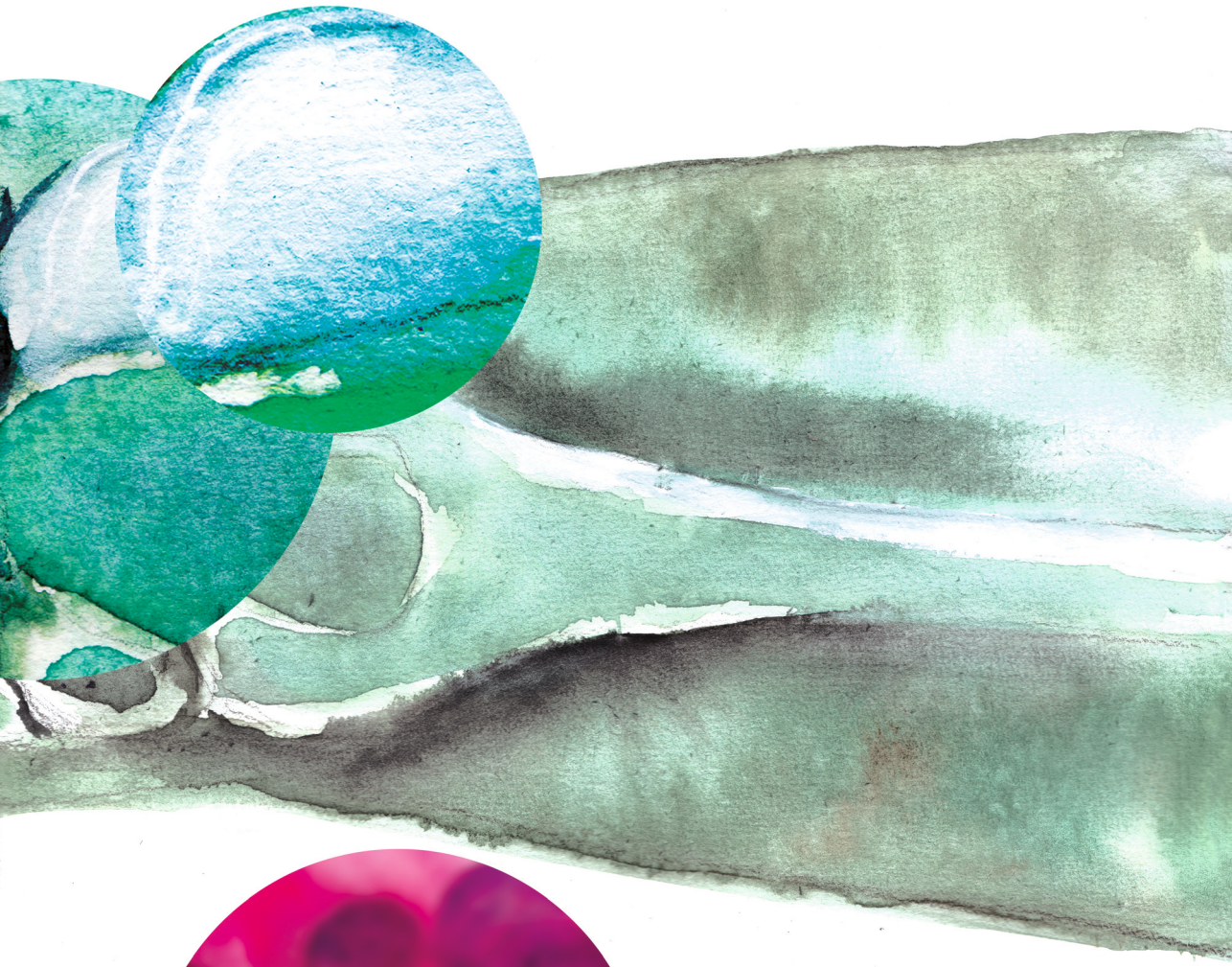
Towards targeting of adaptive immune responses

IN RHEUMATOID ARTHRITIS



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Towards targeting of adaptive immune responses in rheumatoid arthritis

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Towards targeting of adaptive immune responses in rheumatoid arthritis

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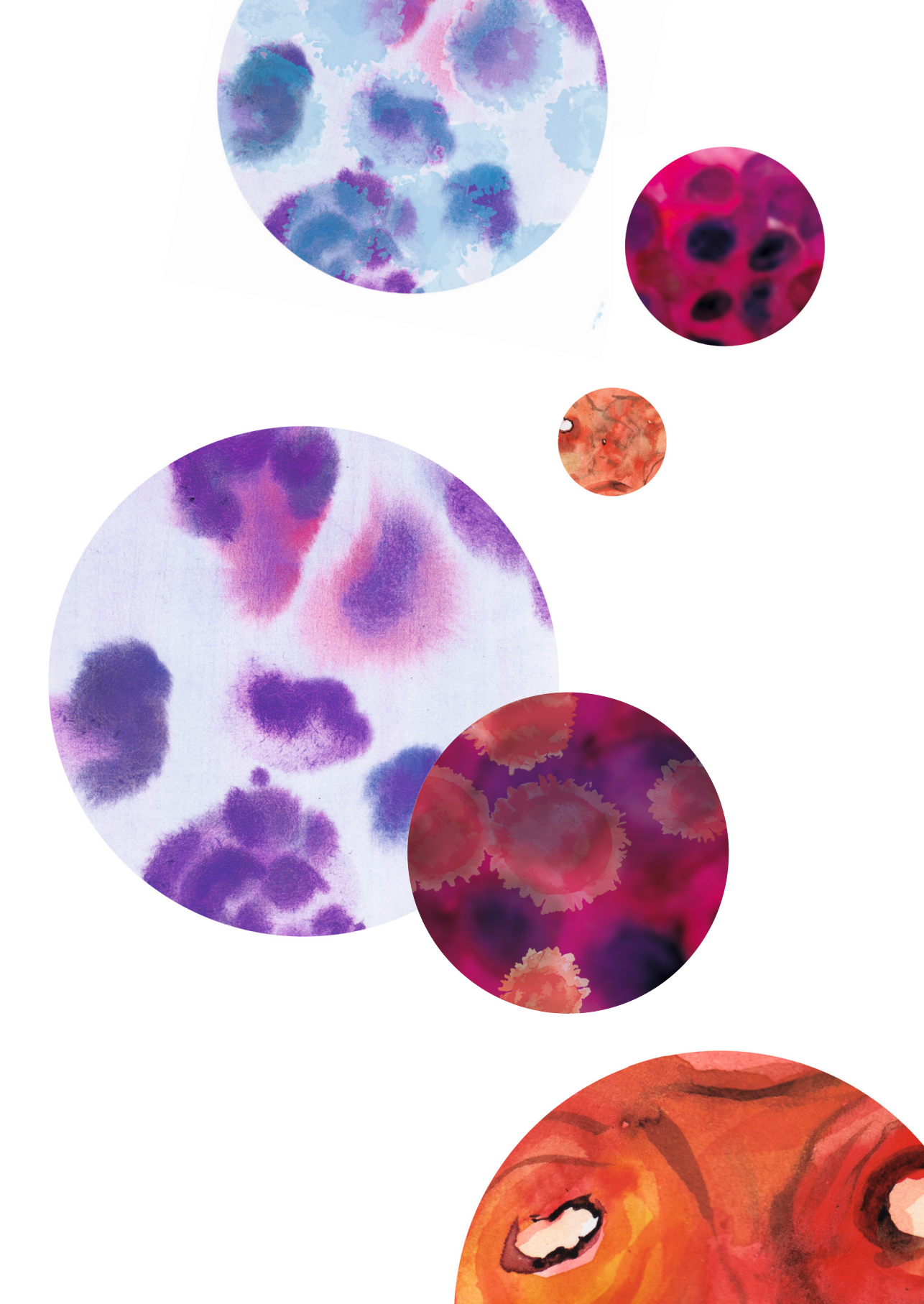

General introduction and outline

1

Rheumatoid arthritis (RA) is a chronic autoimmune disease that affects 0.5-1% of the population worldwide [1,2]. The disease can occur at any age, but on average patients are 40-50 years of age when diagnosed. RA is characterized by symmetrical peripheral polyarthritis, commonly, but not solely, affecting the hands, feet, knees, or ankles. Signs and symptoms of arthritis are usually accompanied by systemic inflammation and other clinical manifestations. Furthermore, RA is associated with an increased risk of cardiovascular disease (i.e. atherosclerosis and vasculitis) and interstitial lung disease [3,4]. If left untreated the inflammatory process eventually results in joint destruction, in some cases resulting in severe disability [5,6].

RA has a big impact on both the affected individual and society. While some patients can reach remission, not all patients respond to treatment. In addition to the disease itself, this can result in loss of work productivity and impairment of social activities. Even those who do respond are subjected to life-long treatment with costly therapies, which poses a substantial (financial) burden on patients and society.

Autoantibodies

RA is a syndrome rather than one uniform disease and two main subtypes of RA can be distinguished based on the presence or absence of autoantibodies against specific antigens. This is referred to as seropositive and seronegative RA, respectively. The most notable autoantibodies in RA are IgM-rheumatoid factor (RF), which is directed against the Fc tail of IgG, and anti-modified protein antibodies (AMPAs), autoantibodies directed against various post-translationally modified proteins. A variety of AMPAs has been identified, of which anti-citrullinated protein antibodies (ACPAs), are the most prevalent. Between seropositive and seronegative RA some remarkable differences can be observed. For instance, in ACPA-positive patients disease onset occurs at a younger age and in these patients a more severe clinical disease can be observed than in ACPA-negative patients [7]. In line with this, both RF-positive and ACPA-positive patients also have lower remission rates after initiation of disease-modifying anti-rheumatic drug (DMARD) treatment and more joint damage, although clinical manifestations at diagnosis are often indistinguishable between seropositive and seronegative patients [8,9].

Stages

In the evolution from healthy to full-blown RA, different disease phases can be discriminated (see also Figure 1):

- an initial at-risk period (with genetic and/or environmental risk factors)

- a phase of clinically silent autoimmunity (e.g. autoantibodies present)
- clinically suspect arthralgia (CSA); i.e. inflammatory joint pain without arthritis
- undifferentiated arthritis (UA); clinically overt arthritis but RA diagnosis not established yet
- RA (early and established) [10,11]

Nonetheless, some phases might not be apparent in all patients [12].

Figure 1 | overview of stages in RA development

As one can imagine, the identification of seronegative at-risk individuals in the pre-clinical stage, thus before the onset of arthritis, is quite challenging. Therefore, most studies investigating this phase focus on seropositive at-risk individuals. In seropositive individuals, this pre-clinical at-risk phase is characterized by immune system activation, including autoantibody production and non-specific musculoskeletal symptoms, mostly arthralgia [13]. Individuals may also experience fatigue, pain, and transient swelling of the joints [14]. Interestingly, signs of systemic inflammation, such as circulating antibodies and high levels of C-reactive protein (CRP), can be found years before the onset of RA and signify an elevated risk of developing RA in the near future [15,16].

Before developing overt clinical arthritis both ACPA-positive and ACPA-negative patients may have a symptomatic stage, which is characterized by the presence of arthralgia and/or subclinical inflammation. In this CSA phase, ACPA-positive and ACPA-negative patients have somewhat different clinical manifestations, with fewer tender joints but more rapid progression to RA in ACPA-positive patients [17].

Diagnosing and classification of rheumatoid arthritis

In daily practice, the diagnosis of RA is made by the judgement of a rheumatologist, rather than by specific diagnostic criteria. This is usually based on the recognition of the clinical symptoms, radiological features, and autoantibody status. For research purposes, the 2010 American College of Rheumatology/European League Against Rheumatism Classification Criteria for RA have been developed (Figure 2) [18].

Target population: Patients who (i) have at least 1 joint with clinical synovitis and (ii) with the synovitis not better explained by another disease.

	Score
A. Joint involvement (tender/swollen)	
1 large joint	0
2-10 large joints	1
1-3 small joints (± involvement of large joints)	2
4-10 small joints (± involvement of large joints)	3
> 10 joints (at least 1 small joint)	5
B. Serology	
Negative RF & ACPA	0
Low-positive RF/low-positive ACPA	2
High-positive RF/high-positive ACPA	3
C. Acute-phase reactants	
Normal CRP & ESR	0
Abnormal CRP & ESR	1
D. Duration of symptoms	
< 6 weeks	0
≥ 6 weeks	1

Add score of categories A-D:

≥ 6/10 = definite RA

Figure 2 | 2010 American College of Rheumatology/European League Against Rheumatism Classification Criteria for RA [18]
RF: IgM-rheumatoid factor, ACPA: antibodies against citrullinated proteins, CRP: C-reactive protein, ESR: erythrocyte sedimentation rate.

Adaptive immune response

Genetic and immunological studies show that cells of the adaptive immune response are involved in the pathogenesis of RA [19–26]. The specificity of this immune response is encoded by rearranged T- and B-cell receptors (TCR and BCR, respectively) expressed by clones of T- and B-lymphocytes, plasmablasts, and plasma cells. In the time preceding disease manifestation (pre-RA) and in the early stages of clinically overt disease, T-cells shift towards a pro-inflammatory phenotype, with an accumulation of expanded T-cells in the synovium and increased levels of serum pro-inflammatory cytokines, including IL-2 [26–29]. B-lineage cells are also altered in pre-RA individuals, with high levels of IgA plasmablasts in the peripheral blood [30]. Moreover, besides ACPA, RA patients often display other AMPAs, for instance against acetylated and carbamylated proteins, closer to disease onset [31,32]. These findings suggest that the immune system is already derailed years before RA onset, which would potentially allow for targeted interventions to prevent or at least delay disease onset.

To investigate the adaptive immune response thoroughly and on a genomic level next-generation sequencing (NGS) technology was developed. This technique makes it able to analyze the repertoire of T- and B-cell receptors individually on the RNA level, in any given bodily compartment, at any given time. Since clones of activated T- and B-cells present identical TCRs and BCRs at their surface, expanded clones can be identified as a deviation in the repertoire, also known as dominant clones or

highly expanded clones. Simply said; the larger the clone, the higher the frequency of the cells that have the same TCR/BCR. Arbitrarily, we marked a clone larger than 0.5% of the total TCR/BCR repertoire as expanded (Figure 3).

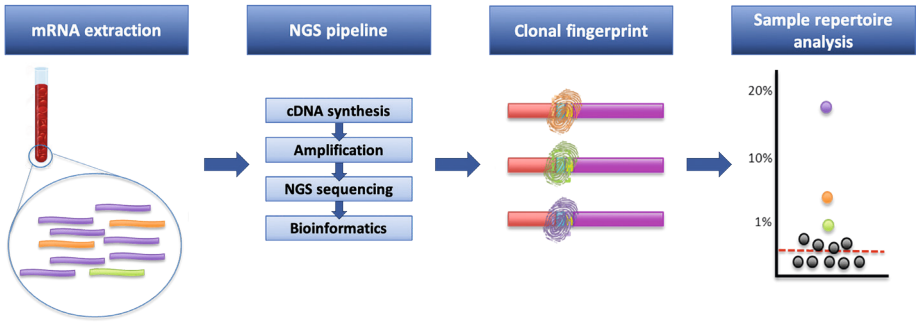

Figure 3 | Next-generation sequencing technology to identify expanded TCR- or BCR-clones

During the first step TCR/BCR mRNA is isolated from a patient sample (i.e. blood, synovial fluid or tissue). From there the next-generation sequencing (NGS) pipeline is started with cDNA synthesis, linear amplification, and sequencing. During bioinformatic analysis unique clones are identified, characterized by fingerprints of their TCR/BCR. In the last step of the bioinformatic process counting of the unique fingerprints yields an estimate of the frequency of each clone. Expanded clones can be identified as a deviation in the repertoire, also known as dominant clones or highly expanded clones (i.e. a clone larger than 0.5% of the total repertoire).

Window of opportunity

In recent years, substantial advances in the treatment of RA have been seen, including the introduction of several new classes of drugs, including biologic Disease-Modifying Antirheumatic Drugs (DMARDs), such as anti-TNF, and targeted synthetic DMARDs (i.e. JAK/STAT-inhibitors). Targeting the adaptive immune response using these targeted therapies, for instance with abatacept (CTLA4-Ig) or rituximab, have been proven to be of clinical benefit in RA patients [24,33]. However, these new therapies are as yet not curative, only effective in 60% of RA patients, and often only induce a partial clinical response [34].

At the moment, no formal treatment recommendations exist for individuals in the pre-clinical stage of RA after presenting with arthralgia; patients are usually monitored over time, but as a rule, they do not receive any (DMARD) treatment until clinical signs of arthritis or a formal diagnosis of RA is established.

However, it has been shown that early treatment initiation in RA patients improves disease signs and symptoms, with lower disease scores and improved physical functioning as well as reduced structural damage detected by radiography [35]. For instance, in the "Prevention of RA by Rituximab" study (PRAIRI study) rituximab significantly delayed RA development, but was unable to prevent the risk of RA development [36]. Early treatment with abatacept showed improvement of subclinical arthritis and reduced RA development [37]. Also, early intervention with methotrexate was only partly successful as it did not prevent RA, but was able to decrease joint inflammation and reduce disease-related symptoms [38]. Many patients also experience a rapid decrease in their workability before treatment initiation and although treatment was unable to reverse disease-associated disability, it stabilized the need for sick leave and prevented incapacity for work [39]. Treatment in the preclinical phase of the disease could lead to fewer complaints in the arthralgia phase, prevention of joint damage, and improved ability to work.

Aim and outline of the thesis

The overarching aim of this thesis is to gain more knowledge on the adaptive immune response in different phases of RA and in various locations; ranging from the early at-risk phase to clinically apparent RA, from studies in blood-only to other bodily compartments, and from T-cells to B-cells.

This thesis consists of three parts: **Part I** of this thesis describes the behaviour of the adaptive immune responses at various sites of inflammation during RA. We aimed to find shared characteristics of the inflammatory process to could give us insight whether development of selective targeting would be an alternative to generalized immunosuppressive strategies. In **Chapter 2**, we use the earlier-mentioned NGS technology to quantitatively assess whether different T-cell clones dominate the inflammatory infiltrate at various sites of inflammation in RA, i.e. in blood, synovial tissue, and synovial fluid. In addition, different joints and different locations within one joint are compared. Furthermore, we analyze to what extent these different compartments share the same dominant T-cell clones. Since T- and B-cells closely interact in adaptive responses, in **Chapter 3** we analyze to what extent different joints also share dominant B-cell clones. This research builds upon the research performed in Chapter 2, by investigating the same compartments but now for the distribution of B-cells.

Part II of this thesis focuses on the earliest stages of RA (i.e. the at-risk phase, phase of clinically silent autoimmunity, CSA, and UA), as early intervention in at-risk

individuals has the theoretical potential to delay or even prevent disease onset. In **Chapter 4**, we perform a systematic literature review in order to provide an overview of all preventive strategies applied to at-risk individuals, taking into account all studies that have hitherto been performed and ongoing clinical trials, as well as patient perspectives to understand the feasibility of these types of interventions.

In **Part III** of this thesis, the behaviour of B-cells before and after B-cell depletion is investigated. To gain more insight in the pathophysiology of B-cells in both RA and at-risk individuals. In **Chapter 5**, we investigate the depletion and repopulation of B-cells after B-cell depletion with rituximab treatment in RA patients. Although B-cell depleting therapy in RA is clearly effective, response is variable and does not always correlate with B-cell depletion itself. Time points of achieved depletion and repopulation are defined based on the percentage of unmutated BCR-clones in the repertoire. Furthermore, the predictive value of early depletion and early repopulation on clinical response is assessed to gain more insight into how this correlates with clinical response. In **Chapter 6**, we focus on B-cells in individuals at-risk of developing RA. We investigate changes in the BCR repertoire over time and the effects of a single dose of rituximab in at-risk individuals. For this, we use data from the earlier mentioned randomized controlled trial, the PRAIRI study, in combination with data from a longitudinal cohort, the "DOMINant clones in the Onset of RA" (DOMINO) study. In addition, a phenotypic analysis of B lineage cells is performed in a similar cohort of at-risk-individuals.

Finally, in **Chapter 7** a summary of the studies presented in this thesis is provided and discussed in light of current literature, with an outlook to future perspectives.

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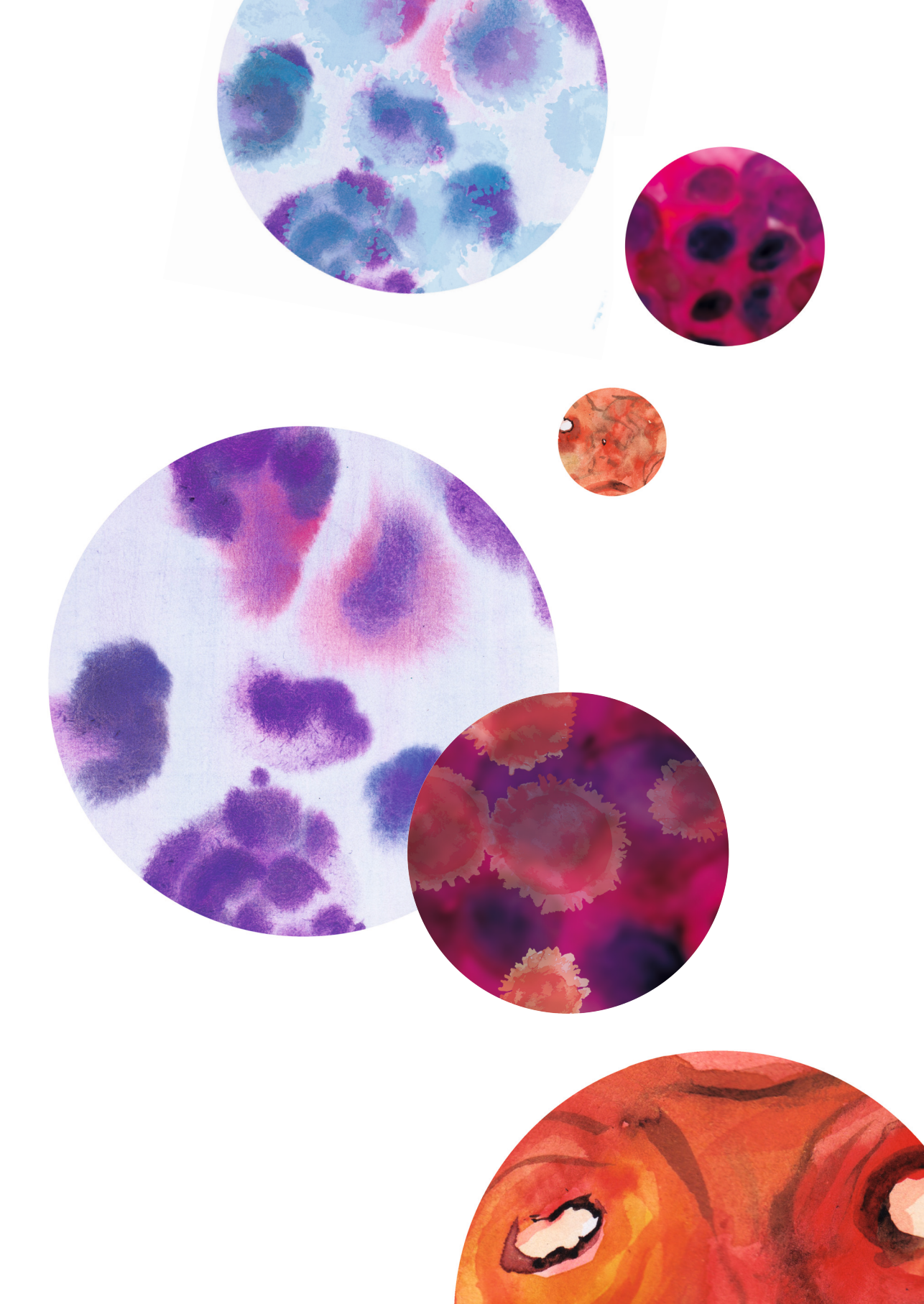
PART I

Adaptive immune responses at sites of inflammation



In rheumatoid arthritis, synovitis at different inflammatory sites is dominated by shared but patient-specific T cell clones

Anne Musters, Paul L. Klarenbeek, Marieke E. Doorenspleet, Giulia Balzaretti, Rebecca E.E. Esveldt, Barbera D.C. van Schaik, Aldo Jongejan, Sander W. Tas, Antoine H.C. van Kampen, Frank Baas, Niek de Vries

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2

Abstract

Genetic and immunological evidence clearly points to a role for T cells in the pathogenesis of rheumatoid arthritis (RA). Selective targeting of such disease-associated T cell clones might be highly effective while having few side effects. However, such selective targeting may only be feasible if the same T cell clones dominate the immune response at different sites of inflammation. We leveraged high-throughput technology to quantitatively assess whether different T cell clones dominate the inflammatory infiltrate at various sites of inflammation in this prototypic autoimmune disease. In 13 RA patients, we performed quantitative next-generation sequencing–based human TCRβ repertoire analysis in simultaneously obtained samples from inflamed synovial tissue (ST) from distinct locations within one joint, from multiple joints, and from synovial fluid (SF) and peripheral blood (PB). Identical TCRβ clones dominate inflammatory responses in ST samples taken from different locations within a single joint and when sampled in different joints. Although overall ST–SF overlap was comparable to higher ST–ST values, the overlap in dominant TCRβ clones in ST–SF comparisons was much lower than ST–ST and comparable to the low ST–PB overlap. In individual RA patients, a limited number of TCRβ clones dominate the immune response in the inflamed ST regardless of the location within a joint and which joint undergoes biopsy; in contrast, there is limited overlap of ST with SF or PB TCR repertoires. This limited breadth of the T cell response in ST of the individual RA patient indicates that development of immunotherapies that selectively modulate dominant T cell responses might be feasible.

Introduction

In recent years, we have seen substantial advances in the treatment of rheumatoid arthritis (RA), including the introduction of several new drugs. However, these new therapies are not curative, are effective in only 60% of the RA patients, and often only induce partial clinical response [1]. Thus, there is a clear need to identify novel, targeted, more effective therapies.

Genetic and immunological studies show that cells of the adaptive immune response are involved in the pathogenesis of RA [2–9]. Specificity of this immune response is encoded by rearranged T and B cell receptors expressed by clones of T and B lymphocytes, plasmablasts, and plasma cells. Targeting the adaptive immune response via biologics, such as abatacept (CTLA4-Ig) and rituximab, has been proven to be of clinical benefit in RA patients [7,10]. Recent advances in selective immunomodulation of disease-associated T and B cell clones resulted in novel, more selective, intensive, and effective therapies in the field of oncology [11,12]. Such Ag receptor–directed therapies would also hold promise for more effective treatment in RA provided that a common signature for RA can be found. However, so far, analyses on Ag receptor characteristics in RA synovial tissue (ST) have yielded two seemingly paradoxical findings: 1) several studies observed sharing of T cell clones between different joints, whereas 2) other studies show that different joints show a huge variation in T cell clones, even within a single patient [9,13–25]. As a consequence, it is unclear whether T cell clones in RA are homogeneous and whether they can be used for targeted therapies.

In this study, we aim to shed more light on this paradox, taking a high-throughput quantitative approach to TCR repertoire analysis in a unique cohort of 13 RA patients in whom synovial biopsy specimens were taken from multiple locations within the same joint, in another (contralateral) joint, as well as in synovial fluid (SF) and peripheral blood (PB) samples. In doing so, we aimed to answer the following three questions: 1) do various clones dominate the T cell response at different locations within one single inflamed joint? 2) do different T cell clones dominate the TCRβ repertoire in multiple joints? and 3) are TCRβ repertoires in ST and SF dominated by different T cell clones?

Materials and methods

Patients

We included 13 RA patients meeting the 2010 American College of Rheumatology/ European League Against Rheumatism Classification Criteria for RA who had active disease (disease activity score evaluated in 28 joints >3.2) [26]. All but one patient were autoantibody positive (anticyclic citrullinated peptide test >25 kAU/l and/or IgM rheumatoid factor >12.5 kU/l). All patients were typed for HLA class II alleles (Supplemental Table 1). Two patients were treated with a biological at the time of arthroscopy (infliximab and rituximab; last infusion 1 month before sampling). We did not observe a significant difference in the number of TCRβ clones, the number of highly expanded TCRβ clones (HECs), or the impact of these HECs on the total TCR repertoire between the different types of treatment (data not shown). More details on patient characteristics are shown in Table 1. From 10 patients, ST biopsy specimens were taken from either one (n = 1) or two inflamed joints (n = 9), all of which were paired with PB. In seven of these patients, we also collected SF from the same joint on which biopsy was performed prior to the arthroscopy. Three additional patients were included for paired SF and PB analysis. The study was approved by the independent Medical Ethics Committee of the Academic Medical Center/University of Amsterdam and performed according to the Declaration of Helsinki. All patients gave written informed consent.

Sampling of synovial biopsy specimens, SF, and PB

To obtain ST biopsy specimens, a minimally invasive arthroscopy was performed from a clinically inflamed knee or ankle, as described previously [27]. ST biopsy specimens from 11 inflamed knee joints were taken from two locations: the infrapatellar (IP) and the suprapatellar (SP) regions. If biopsy was performed on multiple joints, this was done within the same day. SF was obtained by arthrocentesis. In case of a combined collection of ST and SF, the SF was collected prior to the arthroscopy to avoid contamination of SF by hemorrhagic fluid. In all patients, PB was drawn at the time of the arthroscopy and/or arthrocentesis.

Table 1 | Patient characteristics (n=13)

Age (mean (SD)), years	52 (13)
Male (n (%))	3 (23%)
IgM-RF positive (n (%))	12 (92%)
IgM RF level (median (IQR)), kU/L	68 (26-276)
ACPA positive (n (%))	7 (54%)
ACPA level (median (IQR)), kAU/L	84 (8-644)
IgM-RF and ACPA both pos. (n (%))	7 (54%)
Disease duration (median (IQR)), months	7 (3-159)
DAS28 (mean (SD))	5.2 (1.1)
Patients without therapy (n (%))	7 (54%)
Patients treated with csDMARD (n (%))	4 (31%)
Patients treated with bDMARD (n(%))	2 (15%)

IgM-RF, IgM rheumatoid factor; ACPA, anti-citrullinated peptide antibodies; csDMARD, conventional synthetic disease-modifying antirheumatic drugs; bDMARD, biological disease-modifying antirheumatic drugs.

Linear amplification and next-generation sequencing

The protocol used for linear amplification and next-generation sequencing has been described before [9,28,29]. Sequencing was performed on the MiSeq Gene and Small Genome Sequencer (Illumina). Based on earlier studies, TCRβ clones with a frequency ≥0.5% were considered dominant and therefore called HECs [9,30].

Statistics

Values are either expressed as mean or median depending on the presence of a normal or nonnormal distribution of the data. To test for similarity between different sites, the Chao-modified Sørensen index was used. This index, originating from the field of biodiversity, measures dispersion and gives a value between 0 and 1. Values near 0 indicate no overlap between two locations, whereas values close to 1 indicate that the two locations are identical [31–34]. Differences between groups were analyzed using the unpaired Mann–Whitney U test, paired t test, one-way ANOVA, or Tukey multiple comparison test if appropriate. The p values <0.05 (two-sided) were considered statistically significant. R (version 3.1.0), package SpadeR (version 0.1.1), and GraphPad Prism (version 6.0) were used to perform the analyses.

Results

Do various clones dominate the T cell response at different locations within one single inflamed joint?

In large joints, ST biopsy specimens can be obtained from multiple anatomic locations. For example, in the knee, biopsy specimens can be taken from either the SP or IP region. It is unknown whether the T cell repertoires are different at these distinctive locations. To investigate this, we included seven RA patients (see Table 1 for characteristics) with inflamed knee joints and took biopsy specimens from both the SP and IP regions from both inflamed joints (in total, n = 14) and analyzed the TCRβ repertoires. For comparison, we used TCR repertoires from the paired PB samples. We used 26,866 quality-filtered, randomly selected TCRβ sequences per sample. The number of TCRβ clones found in the SP and IP regions (mean 6679 [SD 2612] versus 7869 [SD 2550], respectively) and the number of HECs (mean 8.0 [SD 3.8] versus 7.6 [SD 3.8]) were comparable (Fig. 1A, 1B). The HECs accounted for 29.9% (mean, SD 23.4) and 18.2% (SD 12.8) of the total repertoire, respectively (Fig. 1C). PB showed a similar number of TCRβ clones (9982, SD 5898) with a mean of 6.9 HECs (SD 6.2) that accounted for 40.7% (SD 32.4) of the total repertoire (Fig. 1A–C).

Subsequently, using PB as control, we analyzed to what extent the 25 most expanded (top 25) TCRβ clones in the SP and IP regions showed overlap. Of the 25 most expanded TCRβ clones in the SP region, 54.3% (mean, SD 17.7) were also present among the 25 most expanded TCRβ clones in the IP region. This overlap was significantly lower when comparing the IP samples with PB (mean 20.0%, SD 8.0, $p < 0.0001$; Fig. 1F), leading us to believe that dominant TCRβ clones from the ST are hardly present in the PB. Comparable results were found if we restricted the analysis to the top 10 or top 100 TCRβ clones (Supplemental Fig. 1). Of the top 25 SP TCRβ clones, 86% could be retrieved among the top 1000 of the IP TCRβ clones (Supplemental Fig. 2), showing that the vast majority of the expanded SP TCRβ clones is also present in the IP region.

If we do not focus on the most dominant clones, but instead look at the total measured TCRβ repertoire, we used the Chao-modified Sørensen index to test for similarity between the different regions. For the measured TCRβ repertoires in SP and IP regions, we thus observed a score of 0.49 (mean, SD 0.11), which is significantly higher than that for the comparison of ST and PB (mean 0.15, SD 0.07; $p < 0.0001$) (Fig. 1G). For these analyses, we only included the patients from whom we had a complete dataset, and therefore paired analyses were possible.

Collectively, these findings demonstrate that in the inflamed RA ST, the TCR repertoire shows substantial similarity at different regions within one joint and is dominated by the same TCRβ clones. Such overlap is not observed when comparing ST to PB.

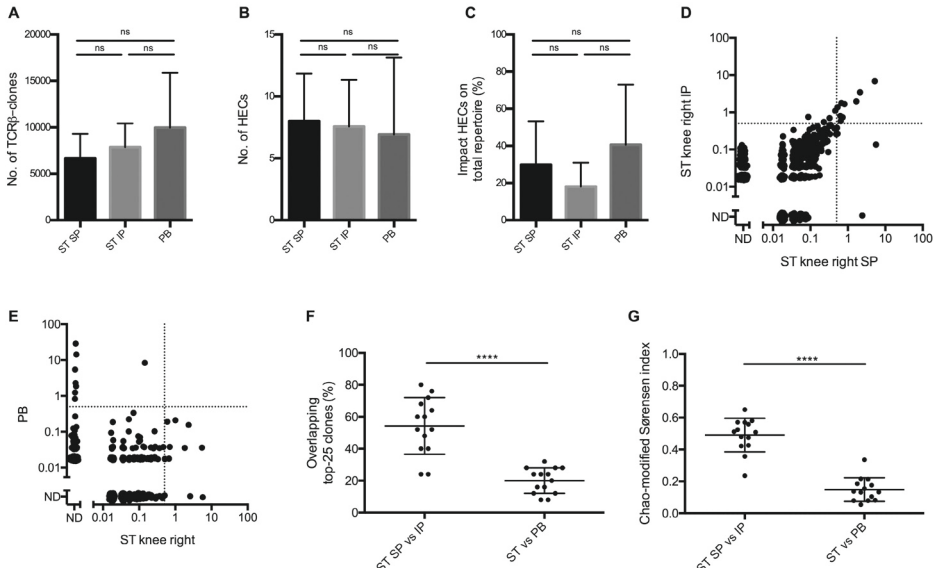

Figure 1 | Comparing T-cell receptor repertoires within one joint
Bar charts of (A) the number of TCRβ-clones, (B) number of highly expanded TCRβ-clones (HECs) and (C) impact of HECs on total repertoire per compartment (bars show mean and SD; using a Tukey's multiple comparison test). (D) Example of overlap-plot from one patient comparing the suprapatellar (SP) to the infrapatellar (IP) synovial tissue (ST) region, showing clear overlap of dominant TCRβ-clones in the upper right quadrant; (E) Comparison of ST to peripheral blood (PB), showing little overlap. Scatter plots of (F) percentage of overlapping top-25 TCRβ-clones and (G) Chao-modified Sørensen indices of the total TCRβ-clones repertoire when comparing different compartments (n=14; lines at mean and SD; **** p<0.0001 using a paired t test).

Do different T cell clones dominate the TCRβ repertoire in multiple joints?
Polyarthritis is a hallmark feature of RA. To the best of our knowledge, no quantitative analysis exists on whether different T cell clones dominate the TCR repertoire in multiple joints. To test this hypothesis, we compared the TCRβ repertoires from ST biopsy specimens simultaneously taken from two inflamed contralateral joints (left and right; either knee or ankle) from nine RA patients (see Table 1 for characteristics). We analyzed paired PB samples as a control. The general features of the ST TCR repertoires from contralateral joints were not significantly different: in the left joint, we identified 7124 TCRβ clones (mean, SD 2665) with 7.1 HECs (mean, SD 2.8); in the right joint, we observed 6735 TCRβ clones (SD 3452) and 9.7 HECs (SD 5.2)

(Fig. 2A, 2B). The impact of the HECs on the total repertoire was 18.9% (mean, SD 14.5) and 18.5% (SD 10.6), respectively, which was also not significantly different (Fig. 2C). Next, we compared the overlap between the 25 most expanded TCRβ clones in both joints. In line with the findings on different locations within the same joint, we again observed that 50.2% (mean, SD 14.9) of the 25 most dominant ST TCRβ clones were identical between different joints (p = 0.30, Fig. 2E). Last, the Chao-modified Sørensen index as a measurement for similarity was assessed. In contrast to what was hypothesized, the Chao-modified Sørensen index also demonstrated large overlap, with a mean score of 0.43 (SD 0.11) (Fig. 2F). Both ST–ST overlap analyses showed significantly higher overlap when compared with overlap between ST and PB (p < 0.001, p < 0.0001, respectively, Fig. 2E, 2F). The Chao-modified Sørensen index between contralateral joints did not differ from the ST overlap observed when comparing two regions within one joint (i.e., IP versus SP [p = 0.12]).

In summary, these data support the notion that TCR repertoires in ST biopsy specimens taken simultaneously from two different (contralateral) inflamed joints show substantial overlap.

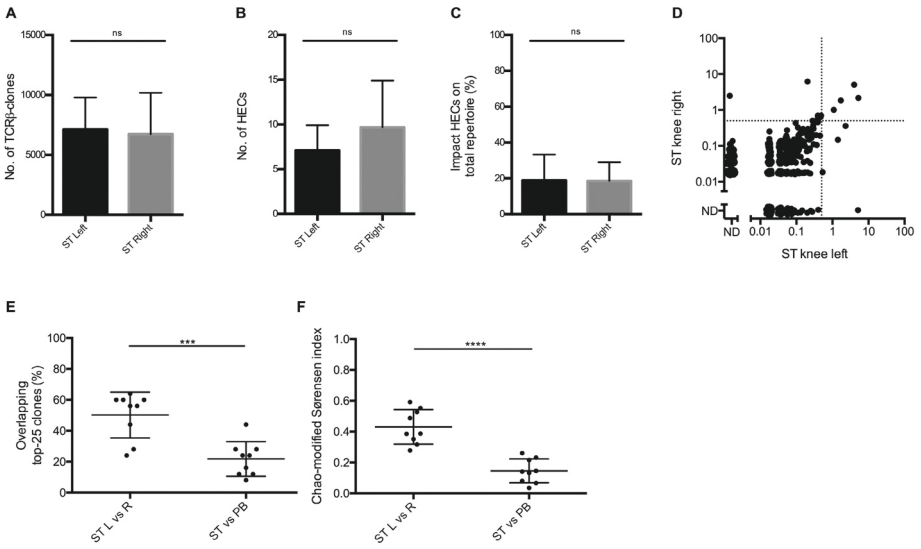

Figure 2 | Comparing T-cell receptor repertoires in two contralateral inflamed joints
Bar charts of (A) the number of TCRβ-clones, (B) number of highly expanded TCRβ-clones (HECs) and (C) impact of HECs on total repertoire per joint (bars show mean and SD; using a one-tailed Mann-Whitney test). (D) Example of overlap-plots from one patient when comparing the ST of the left (L) joint to right (R) joint, showing substantial overlap. Scatter plot of (E) percentage of overlapping top-25 TCRβ-clones and (F) Chao-modified Sørensen indices of the total TCRβ-clones repertoire when comparing different compartments (n=9; lines at mean and SD; *** p < 0.001, **** p < 0.0001 using a paired t test).

Are TCR repertoires in ST and SF dominated by different T cell clones?
Besides synovitis, joint effusion is also a feature of arthritis. As SF can be acquired with a less invasive procedure than ST, fluid is often used to study certain aspects of the pathogenesis of arthritis. However, it is not known whether the T cell repertoires from ST are similar to those from SF in the same joint. To address this, we included seven RA patients (see Table for characteristics) from whom we obtained paired samples: ST biopsy specimens and SF from the same joint as well as PB as control.

In ST, we detected 6781 TCRβ clones (mean, SD 2759) compared with 4923 TCRβ clones in SF (SD 3048, Fig. 3A). We identified 8.2 HECs in ST (mean, SD 4.0) and 18.7 HECs in SF (SD 15.6, $p < 0.001$, Fig. 3B). The HECs accounted, respectively, for 23.0% (mean, SD 18.5) and 45.3% (SD 27.2) of the total repertoire ($p < 0.05$, Fig. 3C). ST and SF differed significantly from PB regarding the number of TCRβ clones (features described earlier in this article; $p < 0.05$, $p < 0.01$, respectively), whereas the number of HECs in ST and SF was significantly different from that in PB (both $p < 0.01$, Fig. 3A–C).

Comparing ST to SF from the same joint, the top 25 overlap was 26.0% (mean, SD 14.8). The top 25 overlap between SF and PB was 18.0% (SD 8.8, Fig. 3F). Thus, the top 25 analysis did not differ significantly between the ST–SF, SF–PB, and ST–PB comparisons. Comparing all TCRβ clones using the Chao-modified Sørensen index for ST to SF resulted in an index of 0.41 (mean, SD 0.13), whereas we noted an index of 0.13 for the SF–PB comparison (SD 0.06, Fig. 3G). The Chao-modified Sørensen index of the ST–SF comparison did significantly differ from the SF– PB and ST–PB comparisons ($p < 0.001$, $p < 0.0001$, respectively). Moreover, within one joint, both results from the top 25 overlap and Chao-modified Sørensen index differed significantly when comparing the intrajoint ST–SF overlap to the intrajoint ST–ST (IP versus SP regions) overlap as described earlier (Fig. 3F, 3G).

In summary, these data show that the dominant TCRβ clones from ST are not fully reflected in SF and even less so in PB. Moreover, significantly less similarity between ST and SF/PB was observed compared with the high similarity seen within ST biopsy specimens taken from different locations in the same joint.

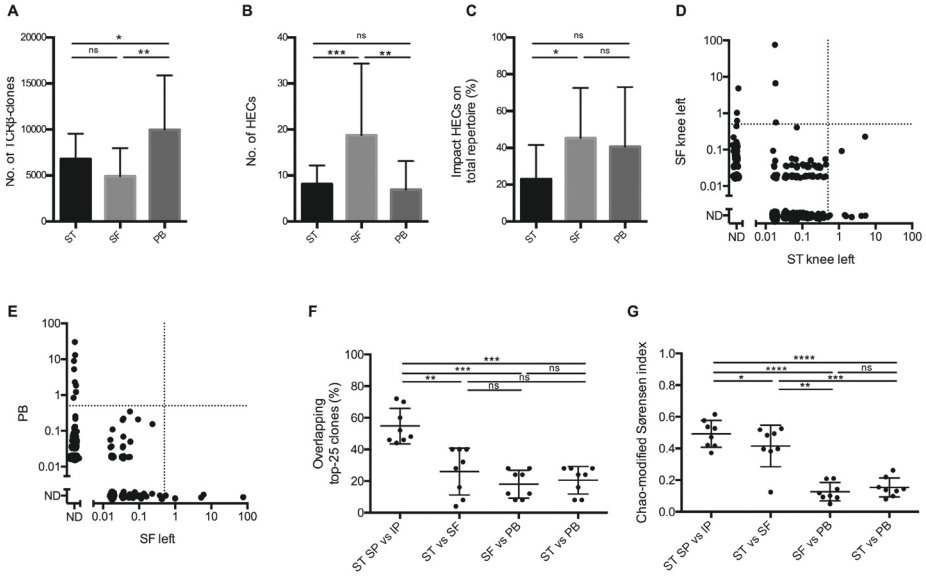

Figure 3 | Comparing T-cell receptor repertoires in synovial tissue, synovial fluid and peripheral blood

Bar charts of (A) the number of TCRβ-clones, (B) number of highly expanded TCRβ-clones (HECs) and (C) impact of HECs on total repertoire per compartment (bars show mean and SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, using a Tukey's multiple comparison test). (D) Overlap-plot comparing synovial tissue (ST) to synovial fluid (SF) in one patient; (E) Overlap plot SF to peripheral blood (PB) in one patient. Scatter plot of (F) percentage of overlapping top-25 TCRβ-clones and (G) Chao-modified Sørensen indices of the total TCRβ-clones repertoire when comparing different compartments (n=8; lines at mean and SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, using a paired t test).

Discussion

This quantitative, comprehensive, whole-repertoire TCR analysis shows that RA synovitis is not dominated by variable, local T cell responses but rather by uniform, systemic T cell responses. Within a single patient, synovial inflammation in multiple joints was dominated by a limited number of expanded TCRβ clones, even when these clones were not dominantly present in PB. This observation suggests that immunotherapy, selectively targeting a limited number of shared, expanded T cell clones, might be effective and feasible [35].

Therefore, to develop this kind of targeted immunotherapy, further characterization of the overlapping "clones" is indicated (e.g., regarding phenotype, TCR α- and β-chain pairing, Ag specificity, and genomic profiles). This meets substantial challenges because it requires harvesting of (enough) cells from the synovium in a phe-

notypically unchanged state, which is currently difficult to perform routinely on a large scale. Hopefully, novel technologies for single-cell characterization will rapidly develop to allow this in the near future.

A striking observation is the difference in TCR repertoire when comparing SF to ST. Previous literature showed an overlap of clones between ST, SF, and PB, but the exact quantitative relation between clones in the different compartments could not be found [36]. An early pilot study already showed that the overlap of TCRβ clones between ST and PB is low (4%) [9]. The present study clearly validates this, but the apparent quantitative difference between TCRβ clones (especially when focusing on the expanded clones) of the SF and ST that we determined was not shown before. Thus, it seems that the ST and SF are two separate compartments instead of one. This is supported by the similarity index, which confirms that the SF shows significantly more overlap with the ST than the PB does. In contrast, the top 25 overlap between ST and SF is as low as the top 25 overlap between ST and PB, indicating that although the general overlap is quite high, this is not the case for the highly expanded ST TCRβ clones. Hence, for future T cell studies in RA, it would be recommended to regard ST and SF as separate compartments and use caution when extrapolating T cell findings from SF to ST.

Our data show clear oligoclonal expansions in the ST and show that the same TCRβ clones dominate the repertoires in biopsy specimens from different regions in the same joint. Even with a very stringent top 25 overlap analysis, we show a large number of overlapping TCRβ clones. These findings imply that single-locus ST biopsies can be used instead of multilocus biopsies when studying T cells. One can therefore speculate that ultrasound (US)-guided or even blind needle biopsies might be just as informative on the T cell repertoire as biopsies taken via arthroscopy. This would improve accessibility because US-guided or blind needle biopsies can be performed at the outpatient clinic, whereas arthroscopy requires theater time. Moreover, these procedures are minimally invasive, well tolerated, and take less time to perform. An earlier study already showed that the quality and RNA yield is preserved in US-guided procedures [37]. Ideally, a follow-up study comparing specimens from US-guided or blind needle biopsies to biopsy specimens obtained via arthroscopy (from the same joint) should be performed to test this.

It should be noted that we sequenced the TCR β-chain only; thus, it remains possible that the identified sequences correspond to “public” sequences (i.e., clones that have the same TCR β-chain but have a different TCR α-chain). This might or might not drive a similar Ag specificity of the receptor depending on the TCR α-chain it is

paired with. Because our studies are based on next-generation sequencing analysis of TCR mRNA, our results might be influenced quantitatively by differences in expression among different types of T cells [9]. However, in vitro studies showed that TCR surface expression and TCR mRNA levels are equal for naive and memory CD4 and CD8 T cells, whereas effector T cells were shown to have at most 2-fold higher levels of TCR mRNA and TCR surface expression, indicating that potential bias in RNA-based TCR studies is probably minimal [9,38,39]. Theoretically, there is a risk of amplification bias due to the fact that some TCR primers might be more efficient than others. However, our system uses an initial linear amplification procedure to prevent this. Furthermore, recent analysis using unique molecular identifiers in our protocol showed excellent correspondence with the results of our linear amplification protocol (data not shown).

The reported findings may potentially be extended to other forms of arthritis. In a pilot study in psoriatic arthritis (PsA) patients, we earlier showed substantial overlap in TCR repertoire between ST in both knees in one PsA patient, whereas overlap between synovial repertoires and PB was limited in two PsA patients [40]. These preliminary results are comparable to the results shown in this study and support the notion that TCRβ clones in ST are different compared with PB in both diseases. Clearly, the findings in PsA need confirmation in larger patient groups.

Follow-up studies on B cell receptor clonality in both diseases and in other immune-mediated inflammatory diseases may shed more light on the composition of the B cell repertoire in different compartments and different phases of the disease. In RA, this would follow up on an earlier study that showed dominant B cell clones shared among multiple joints of the same patient but not in PB [30].

In conclusion, we show substantial overlap of the TCR repertoires between inflamed ST biopsy specimens from different joints or from different sites within one joint in the same patient, whereas SF does not fully reflect the T cell repertoire in the inflamed ST. This implies that for T cell (receptor) analysis, tissue biopsies are required but that tissue collection might be simplified using less invasive procedures [e.g., through high-quality, US-guided, or blind needle biopsies [27,37]. More importantly, it shows that underlying T cell responses in the ST share a uniform specificity, which suggests that Ag- and/or receptor-specific therapies targeting a limited set of TCRβ clones in individual patients may be feasible in RA and possibly other inflammatory joint diseases, including PsA or even the immune-mediated inflammatory disease group at large.

Supplementary data

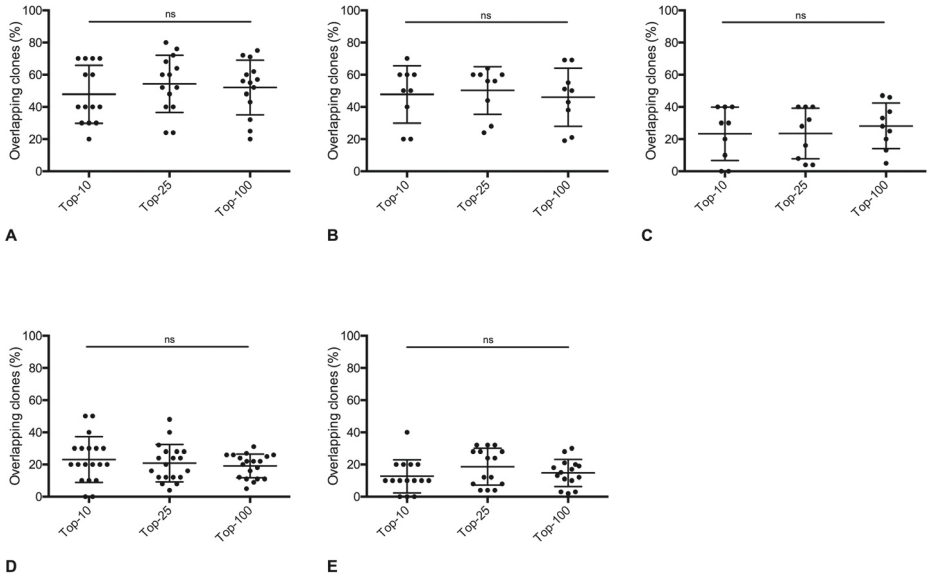

Figure S1 | Top-10, top-25 and top-100 most expanded TCRβ-clones when comparing samples
from (A) within one joint (n=7), (B) two inflamed joints (n=9), (C) synovial tissue (ST) and synovial fluid (SF) (n=7), (D) ST and peripheral blood (PB) (n=10), and (E) SF and PB (n=10) (lines at mean and SD; testing using a one-way ANOVA).

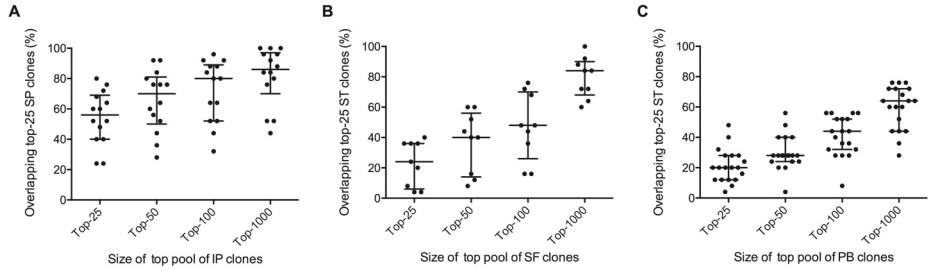

Figure S2 | Percentage of overlapping top-25 TCRβ-clones
from the (A) suprapatellar (SP) region in comparison to various sizes of top pools of infrapatellar (IP) TCRβ-clones (n=14), (B) synovial tissue (ST) in comparison to various sizes of top pools of synovial fluid (SF) TCRβ-clones (n=9), (C) ST in comparison to various sizes of top pools of peripheral blood (PB) TCRβ-clones (n=19).

Table S1 | HLA typing

Patient	HLA-DPB1		HLA-DQB1		HLA-DRB1	
1	04:01:01	04:01:01	02:01:01	02:01:01	07:01:01	03:01:01
2	05:01:01	04:01:01	05:03:01	03:03:02	09:01:02	14:04
3	02:01:02	04:01:01	05:03:01	03:02:01	04:01:01	14:54:01
4	17:01	04:01:01	06:02:01	03:03:02	07:01:01	15:01:01
5	04:01:01	04:01:01	05:01:01	03:01:01	01:01:01	04:01:01
6	14:01	04:02:01	03:02:01	05:01:01	01:01:01	04:01:01
7	14:01	02:01:02	05:02:01	03:02:01	04:02:01	16:01:01
8	02:01:02	04:01:01	05:01:01	03:01:01	10:01:01	04:01:01
9	02:01:02	04:01:01	05:03:01	06:01:01	15:02:01	14:04
10	02:01:02	03:01:01	05:02:01	03:01:01	01:01:01	11:01:01
11	05:01:01	01:01:01	05:02:01	05:02:01	16:02:01	15:01:01
12	03:01:01	04:01:01	06:02:01	03:02:01	04:01:01	15:01:01
13	03:01:01	03:01:01	06:03:01	03:01:01	04:01:01	13:01:01

Six-digit HLA typing of all included patients (n=13) was performed using RNA-sequencing [41,42].

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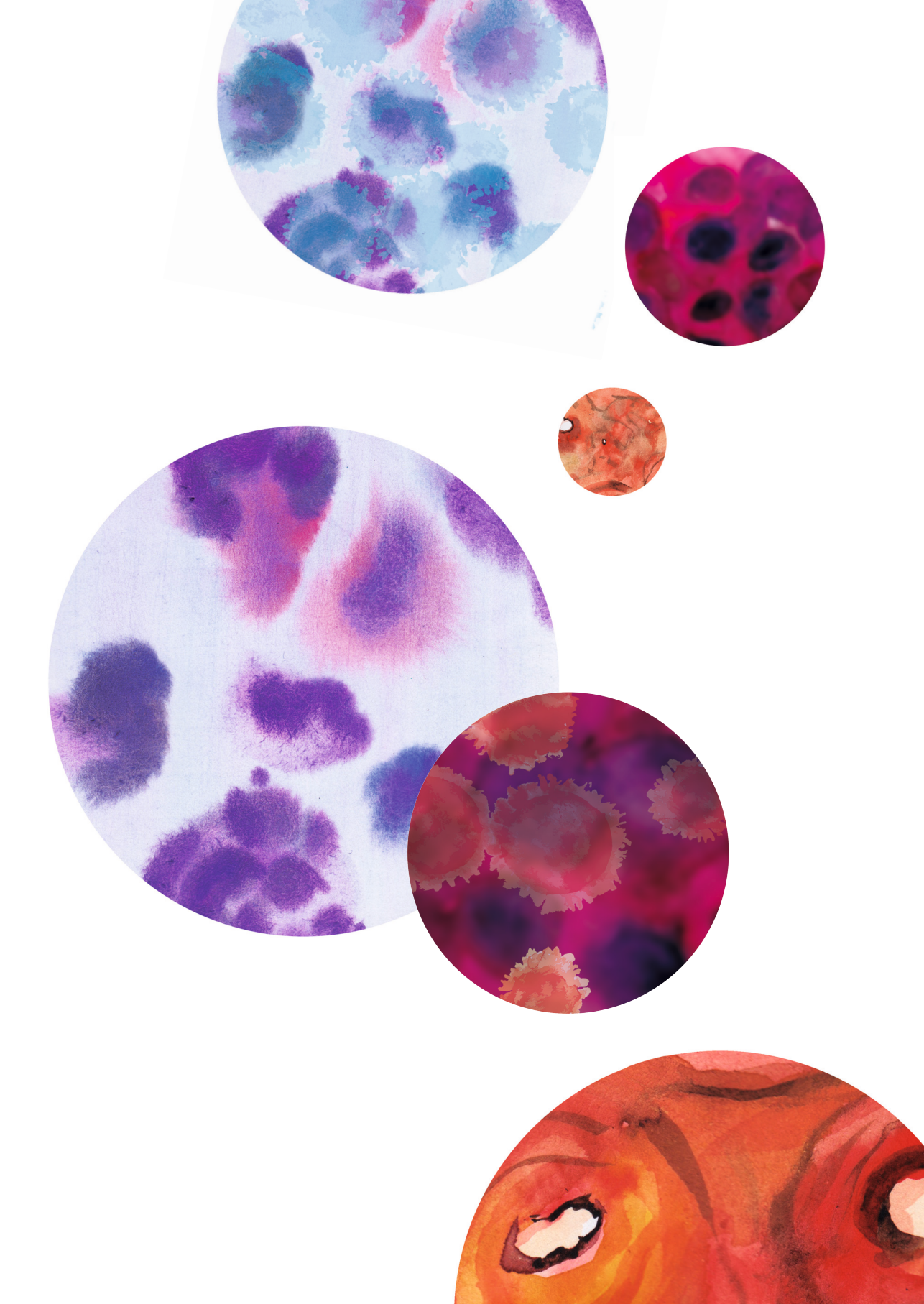

In rheumatoid arthritis inflamed joints share dominant patient-specific B-cell clones

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3

Abstract

Background In patients with rheumatoid arthritis (RA) different joints were shown to share the same dominant T-cell clones, suggesting shared characteristics of the inflammatory process and indicating that strategies to selectively target the antigen receptor might be feasible. Since T- and B- lymphocytes closely interact in adaptive responses, we analysed to what extent different joints also share dominant B-cell clones.

Methods In 11 RA patients, quantitative B-cell receptor (BCR) repertoire analysis was performed in simultaneously obtained samples from inflamed synovial tissue (ST) from distinct locations within one joint, from multiple joints, from synovial fluid (SF) and peripheral blood (PB).

Results ST biopsies from different locations in the same joint showed clear overlap in the top-25 dominant BCR clones (16.7%, SD 12.5), in the same range as the overlap between ST and SF in the same joint (8.0%, SD 8.8) and the overlap between ST-ST between different joints (9.1%, SD 8.2), but clearly higher than the overlap between ST and PB (1.7%, SD 2.4; $p<0.05$) and SF and PB (2.7%, SD 4.1; $p<0.05$). Interestingly, these figures were substantially lower than the overlap observed in previous T-cell clonality studies.

Conclusions We conclude that in RA BCR clonal responses may be more localized than TCR clonal responses, pointing to antigen-selective influx, proliferation and/or maturation of B-cells. B lineage cells in the SF may adequately represent the dominant BCR clones of the ST, which is in contrast to T-cells. Collectively, the presence of shared B- and especially T-cells in different joints from the same patient suggests that approaches might be feasible that aim to develop antigen-receptor specific targeting of lymphocyte clones in RA as an alternative to more generalized immunosuppressive strategies.

Introduction

Adaptive immune cells are key players in the pathogenesis of rheumatoid arthritis (RA) [1–8]. The immune response in RA is encoded by rearranged receptors expressed by clones of T and B lymphocytes, plasmablasts and plasma cells. Targeting the adaptive immune response using novel targeted therapies, also known as biologics, for instance with abatacept (CTLA4-Ig) or rituximab has been proven to be of clinical benefit in RA patients [6,9]. Despite these important developments in the treatment of RA, there is still no cure for RA patients available. Only 60% of RA treatments are effective, and often merely induce a partial clinical response [10]. Thus, there is a clear need to develop novel, targeted, more effective therapies.

Recent studies showed that a common T-cell receptor signature can be found in the synovial tissue (ST) of RA patients [11]. This suggests that antigen- and/or receptor-specific therapies targeting a limited set of T-cell clones in individual patients may be feasible in RA. Another key player of the adaptive immune system, the B-cell, also appears to be of great relevance in RA as it is an antibody-driven disease in which anti-cyclic citrullinated peptide antibodies (ACPA) and IgM- rheumatoid factor (IgM-RF) play a key role. Consequently, antigen receptor specific targeting of B-cells might even be more attractive. However, to assess whether B-cells might indeed constitute an interesting target for antigen receptor specific therapy, more information on the distribution of B- cell clones over different joints in the same patient is required.

This study was designed to investigate the distribution of the B-cell receptor (BCR) repertoire using a high-throughput quantitative approach in a unique cohort of 11 RA patients, in which synovial tissue (ST) biopsies were taken from multiple locations within the same joint, in another (contralateral) joint, as well as synovial fluid (SF) and peripheral blood (PB) samples. Using this method, we aimed to answer the following three questions: 1) Do different B-cell clones dominate the BCR repertoire at different locations within one single inflamed joint? 2) Are BCR repertoires in different inflamed joints dominated by the same BCR-clones? 3) Do the same dominant BCR-clones reside in different compartments, such as ST, SF and PB at the same time?

Materials and methods

Patients

Eleven RA patients meeting the 2010 ACR/EULAR Classification Criteria for RA with active disease (disease activity score evaluated in 28 joints (DAS28) >3.2) were included [12]. Details of the included patients are described earlier [11]. All were autoanti-

body positive (anti-cyclic citrullinated peptide test >25 kAU/l and/or IgM-rheumatoid factor >12.5 kU/l). Two patients (patients 3 and 11) were treated with a biological at the time of arthroscopy, i.e. rituximab and infliximab (both last infusion 1 month before sampling). More details on patient characteristics are shown in Table 1. From 7 patients synovial tissue biopsies were taken from two inflamed joints, all paired with peripheral blood. From 4 of these patients, we also collected synovial fluid from the biopsied joint prior to the arthroscopy. Three additional patients were included for paired SF and PB analysis. The study was approved by the independent Medical Ethics Committee of the Amsterdam University Medical Center/University of Amsterdam and performed according to the Declaration of Helsinki. All patients gave written informed consent.

Table 1 | Patient characteristics (n=11)

Age (mean (SD)), years	53 (13)
Male (n (%))	2 (18%)
IgM-RF positive (n (%))	11 (100%)
IgM RF level (median (IQR)), kU/L	68 (33-322)
ACPA positive (n (%))	6 (55%)
ACPA level (median (IQR)), kAU/L	84 (8-499)
IgM-RF and ACPA both pos. (n (%))	6 (55%)
Disease duration (median (IQR)), months	45 (3-192)
DAS28 (mean (SD))	5.2 (1.2)
Patients without therapy (n (%))	7 (64%)
Patients treated with csDMARD (n (%))	4 (36%)
Patients treated with bDMARD (n(%))	2 (18%)

IgM-RF, IgM rheumatoid factor; ACPA, anti-citrullinated peptide antibodies; csDMARD, conventional synthetic disease-modifying antirheumatic drugs; bDMARD, biological disease-modifying antirheumatic drugs.

Sampling of synovial biopsies, synovial fluid and peripheral blood

To obtain ST biopsies a minimally invasive arthroscopy was performed from a clinically inflamed knee or ankle, as described previously [13]. From 12 out of the 15 inflamed knee joints biopsied, ST biopsies were taken from two locations, the infrapatellar (IP) and the suprapatellar (SP) region. In all patients who underwent arthroscopy, both knee joints where biopsied for ST on the same day. SF was obtained by arthrocentesis. In case of a combined collection of ST and SF, the SF was collected

prior to the arthroscopy in order to avoid contamination of SF by hemorrhagic fluid. In all patients, peripheral blood was drawn at the time of the arthroscopy and/or arthrocentesis.

Linear amplification and next-generation sequencing

The protocol used for linear amplification and next- generation sequencing of the BCR-heavy chain (BCR-heavy) was based on previously described methods [8,11,14–16]. Sequencing was performed on the MiSeq (Illumina). To identify and quantify separate BCR-clones more accurately unique molecular identifiers (UMI) were used [17]. A unique clone was identified by the unique sequence of the CDR3-region in combination with the use of the (V)ariable and (J)oining genes. Based on earlier studies BCR-clones with a frequency ≥ 0.5% were considered dominant, and therefore called Highly Expanded Clones (HECs) [8,18]. BCR repertoire raw fast data are available on the Sequence Read Archive with the accession number BioProject PRJNA822925.

Bioinformatics and statistics

Values are either expressed as mean or median depending on the presence of a (non)-normal distribution of the data. For each sample an equal number of reads (N=9,736) was randomly drawn from the acquired reads to standardize comparisons. The Chao- modified Sørensen index was used to test for similarity between different samples. This biodiversity index measures dispersion and gives a value between 0 and 1. Values near 0 indicate no overlap between two locations, while values close to 1 show that the two repertoires are identical [19–22]. Because somatic hypermutation causes a broader, and more diverse repertoire we decided to only use the CDR3-sequence for the similarity analysis of the top clones and total repertoire. Differences between groups were analyzed using the unpaired Mann–Whitney U test, paired t test, one-way ANOVA or Tukey's multiple comparison test where appropriate. p Values < 0.05 (two-sided) were considered statistically significant. R (version 3.5.1), package SpadeR (version 0.1.1) and Graphpad Prism (version 8.0) were used to perform the analyses.

Results

Different locations within an inflamed joint share dominant BCR clones

When performing an arthroscopy the knee joint can be divided into two anatomic locations, the suprapatellar (SP) and infrapatellar (IP) region. For T-cells we know that synovial tissue biopsies from different locations within the same joint show substantial clonal overlap [11]. However, for B-cells this is still unknown. In this study

we took biopsies from these distinct two regions from 12 different inflamed knee joints in 7 individual RA patients.

The ST biopsies were analyzed for BCR repertoires and compared with paired peripheral blood (PB) samples. The SP and IP regions showed comparable numbers of BCR-clones (mean ± SD: 1,337 ± 651 versus 1,420 ± 552 respectively) and highly expanded BCR-clones (HECs; mean ± SD: 29.7 ± 6.7 versus 32.2 ± 8.9), with a comparable impact of these HECs on the total repertoire (mean ± SD: 55.1% ± 18.0 versus 50.8% ± 17.9). In contrast, PB showed a significantly higher number of BCR-clones ($p < 0.0001$; 5,407 ± 2,533), a lower number of HECs ($p < 0.0001$; 5.3 ± 5.2), and these HECs accounted for significantly less impact on the total repertoire ($p < 0.0001$; 15.6% ± 24.1) (Figures 1A–C).

In order to determine similarity between different BCR repertoires we compared the most expanded BCR-clones between the two biopsied regions, using PB as control. Of the 25 most expanded BCR-clones in the SP region on average 16.7% (mean, SD 12.5) were also present in the top-25 clones in the IP region ("Clonal retrieval"). However, significantly fewer of the expanded BCR clones in SP ($p<0.001$; 1% ± 2.5%) and IP ($p<0.01$; 2% ± 2.7%) could be retrieved in PB samples (Figures 1D–F).

To analyze similarity for the complete BCR, rather than the most dominant clones only, we use the Chao-modified Sørensen index [11]. This index gives an estimate for the combined frequency of all shared clones while correcting for shared clones not observed due to incomplete sampling. The comparison of the SP and IP region showed a mean similarity index of 0.32 (SD 0.17; n=12), significantly higher than the score of 0.06 observed when comparing ST-SP or ST-IP with PB (mean, SD 0.08 and 0.07 respectively, n=12; $p<0.001$, Figure 1G).

Thus, the BCR repertoire in inflamed ST of RA patients shows similarity at different locations within same joint, and shows sharing of some dominant BCR clones, whereas hardly any overlap was observed when comparing ST to PB.

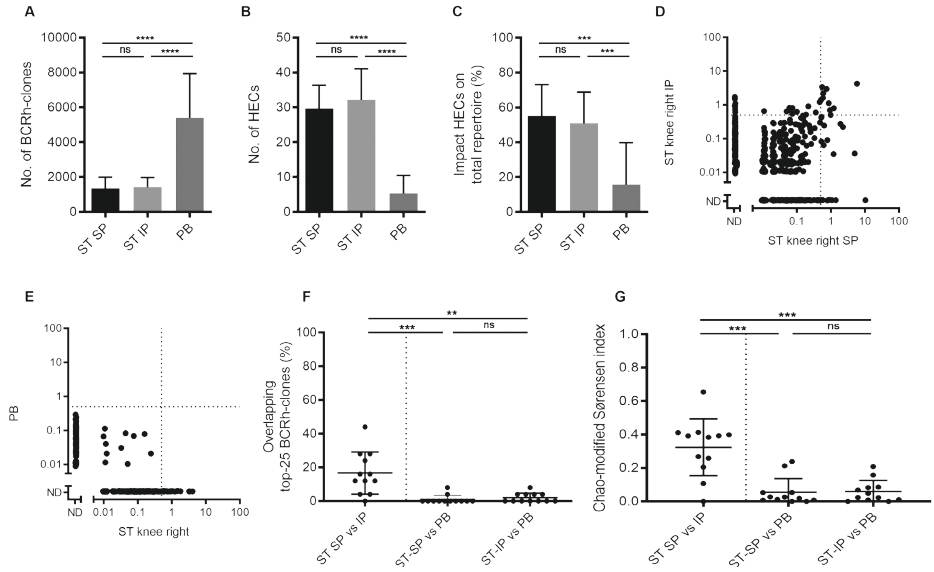

Figure 1 | Comparing B-cell receptor repertoires within one joint
Bar charts of (A) the number of BCR-clones, (B) number of highly expanded BCR-clones (HECs) and (C) impact of HECs on total repertoire per compartment (bars show mean and SD; ** p<0.01 and **** p<0.0001 using a Tukey's multiple comparison test; ns=not significant). (D) Representative example of overlap-plot in one patient comparing the suprapatellar (SP) to the infrapatellar (IP) synovial tissue (ST) region, showing overlap of dominant BCR-clones in the upper right quadrant. Each dot reflects the frequency of the clone (% of total number of reads) in the ST-IP on the Y-axis and in the ST-SP on the X-axis. Vertical and horizontal dotted lines indicate the 0.5% threshold for HECs. ND=not detected; (E) Representative example of a comparison of ST to peripheral blood (PB), showing little overlap. For explanation of graph see (D) above. Scatter plots of (F) percentage of overlapping top-25 BCR-clones and (G) Chao- modified Sørensen indices of the total BCR-clones repertoire when comparing different compartments (n=12; lines at mean and SD; * p<0.05, ** p<0.01 using a paired t test; ns=not significant).

BCR repertoires in different inflamed joints share distinct dominant BCR-clones

To test for sharing of clones between different joints in 7 RA patients the BCR repertoire was compared between ST biopsies from inflamed contralateral knee joints in the same patient that were taken on the same day. Again, paired PB samples from the same time-point served as a control. The overall repertoire characteristics from contralateral joints were not significantly different (Figures 2A–C): In the left knee on average 1,269 ± 616.5 BCR-clones, with 30.4 ± 8.6 HECs accounting for 55.8% ±

19.5% of the total repertoire, in the right knee 1,701 ± 439.7 BCR-clones, with 31.7 ± 10.3 HECs accounting for 46.7% ± 13.9 of the total repertoire.

To test for repertoire similarity between different joints we compared the overlap between the 25 most expanded BCR- clones in both knee joints. The overlap of the top-25 between two different joints was 9.1% ± 8.2, significantly larger than the top-25 overlap between ST and PB (1.7% ± 2.4; p = 0.02; Figures 2D, E). Comparing the total repertoires, the mean Chao-modified Sørensen index was 0.24 ± 0.15 for the ST-ST comparison, significantly higher than the index between ST and PB (0.05 ± 0.06; p < 0.01; Figure 2F). Both the top-25 overlap and Chao-modified Sørensen index between contralateral joints did not differ from the overlap observed when comparing two ST- regions within one joint (p = 0.08 and p = 0.36 respectively).

Together, these data demonstrate that the BCR repertoire in ST from the knee shows substantially more overlap with the repertoire in ST of the contralateral knee than with PB.

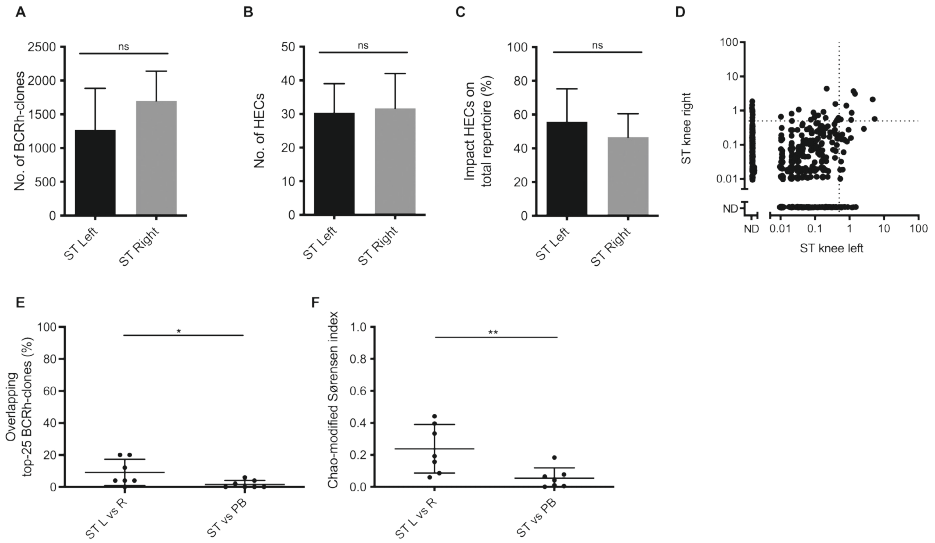

Figure 2 | Comparing B-cell receptor repertoires in two contralateral inflamed joints
Bar charts of (A) the number of BCR-clones, (B) number of highly expanded BCR-clones (HECs) and (C) impact of HECs on total repertoire per joint (bars show mean and SD; ns=not significant using a one- tailed Mann-Whitney test). (D) Representative example of overlap-plots from one patient when comparing the ST of the left (L) joint to right (R) joint, showing substantial overlap; for explanation see legend Figure 1D (E) Scatterplot of the impact of the top-25 overlapping BCR-clones and (F) the Chao-modified Sørensen indices of the total BCR-clonal repertoire when comparing different compartments (n=7; lines at mean and SD; * p<0.05, ** p<0.01 using a paired t test).

BCR-clones that are dominant in ST are shared with SF and to a lesser extent with PB

The pathogenesis of arthritis is often studied by looking into the SF rather than ST. However, until recently, it was still unclear whether the adaptive immune responses in both compartments was similar. We have learned from an earlier study on T-cells that the dominant TCRb-clones in ST are not fully reflected in SF and even less so in PB [11]. However, for B-cells this was not investigated before. Therefore, we included 6 RA patients from whom we obtained simultaneously obtained samples: ST biopsies and SF from the same joint, paired with PB as control.

In ST and SF we detected a comparable number of BCR- clones (1,412 ± 598 vs. 2,188 ± 1,385) and HECs (30.4 ± 8.0 vs. 23.2 ± 12.8; Figures 3A, B). However, the impact of the HECs on the repertoire was significantly higher for ST compared to SF ($p < 0.01$; 53.1% ± 17.3 versus 31.7% ± 19.2; Figure 3C). ST and SF differed significantly from PB regarding the number of BCR- clones and HECs (both $p < 0.0001$), while the impact of HECs on the total repertoire was only significantly different between ST and PB ($p < 0.0001$; Figures 3A–C).

The top-25 overlap in BCR clones between SF and ST from the same joint was 8.0% ± 8.8, significantly higher than the 2.7% ± 4.1 top-25 overlap between SF and PB ($p < 0.05$; Figures 3D–F). This top-25 overlap did not show significant differences in the comparisons ST-SF vs. ST-PB, or SF-PB vs. ST- PB. However, comparing all BCR-clones the Chao-modified Sørensen index was 0.20 ± 0.13 for the SF-ST overlap, significantly higher than the index of 0.02 ± 0.02 for the SF- PB comparison and 0.05 ± 0.06 for the ST-PB comparison (both $p < 0.01$; Figure 3G). Of note, the intrajoint overlap scores did not significantly differ between ST-ST and ST-SF, not when using the top-25 overlap and neither when using the Chao- modified Sørensen index (Figures 3F, G).

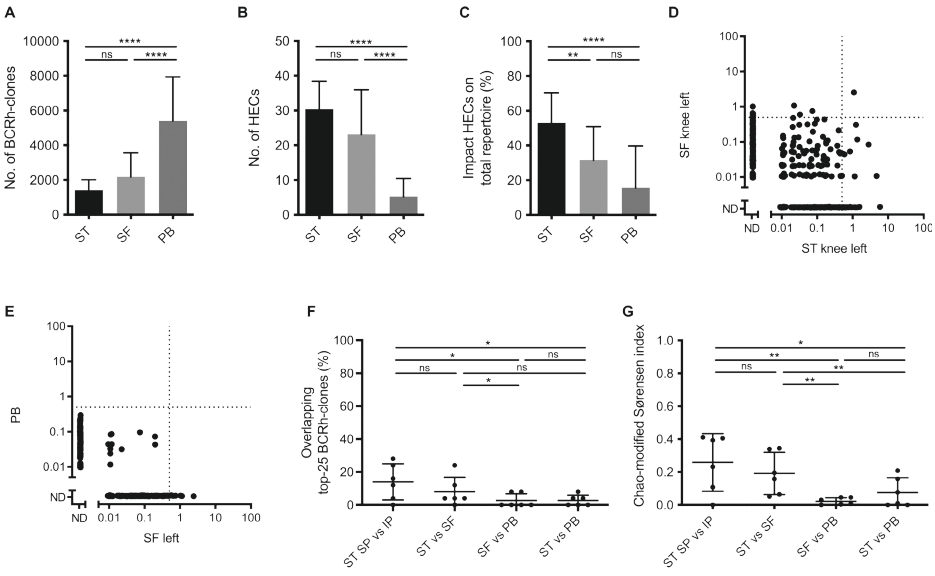

Figure 3 | Comparing B-cell receptor repertoires in synovial tissue, synovial fluid and peripheral blood

Bar charts of (A) the number of BCR-clones, (B) number of highly expanded BCR-clones (HECs) and (C) impact of HECs on total repertoire per compartment (bars show mean and SD; ** p<0.01, **** p<0.0001, using a Tukey's multiple comparison test; ns=not significant). (D) Representative overlap-plots in one patient comparing the synovial tissue (ST) to synovial fluid (SF) and (E) comparing the SF to peripheral blood (PB) in one patient; for further details see legend Figure 1D. Scatter plot of (F) percentage of overlapping top-25 BCR-clones and (G) Chao-modified Sørensen indices of the total BCR-clones repertoire when comparing different compartments (n=6; lines at mean and SD; ** p<0.01, *** p<0.001 using a paired t test; ns=not significant).

Discussion

Using quantitative, B-cell receptor repertoire analysis we demonstrate that RA synovitis in different joints shares dominant B-cell responses. Within the same patient a limited number of expanded B-cell receptor clones were retrieved in the inflamed synovial tissue and fluid in different joints. The observed sharing between synovial tissue in different joints suggests that immunotherapy, selectively targeting a limited number of shared, expanded B-cell clones might be feasible and effective [23].

However, compared to the top-25 overlap observed in T-cell clones within and between synovial tissue samples taken in the same (54%) and in different joints (50%) in a previous study, the overlap in the B-cell compartment is much less pronounced (17% and 9%, respectively) [11]. This is even more clear in the comparison of synovial

tissue with peripheral blood (20% for T- cell and 2% for B-cell clones). This suggests that B-cell responses might be much more localized than T-cell responses. This might suggest localized proliferation and/or maturation with an increase in BCR expression, e.g. in the transformation of mature B-cells to plasma cells. Another explanation could be an antigen-driven influx of specific B-cell clones. Such a localized influx of B-cells into the joint on a stable TCR background might result in a clinical arthritis. It would be very interesting to further investigate this into more detail, e.g. in a prospective study in seropositive individuals at increased risk of RA while sampling paired synovial tissue, fluid and blood samples during the course of the disease.

It is remarkable that in the BCR repertoire the observed ST- SF overlap (8%) is actually in the range of ST-ST overlap within and between different joints (resp 17% and 9%), while the earlier study for T-cell overlap demonstrated a significantly higher ST-ST overlap than SF-ST overlap ($p < 0.01$; 54% and 50% vs 26%, respectively). This might indicate that there is more circulation of B-cells than T-cells from the synovium to the synovial fluid in the B-cell compartment than in the T-cell compartment. Since intra-joint overlaps observed in the ST-SF and ST-ST comparisons are in the same (albeit low) range, our data suggest that B cells derived from synovial fluid or alternatively from synovial tissue are equally informative regarding antigen specificity of B-cells in the synovium. This indicates that for future B-cell studies in RA one might use SF from arthrocentesis as a substitute for ST obtained by arthroscopy or ultrasound- guided biopsy. The latter may have several advantages since arthrocentesis is a less invasive, patient-friendly procedure, which does not require theatre time.

More studies are required to further characterize these overlapping TCR and BCR clones. This might entail functional characterization, including antigen specificity, in order to develop antigen receptor directed therapies. However, it might also concern genomic and proteomic analyses in order to identify more uniform cellular targets selectively present in the activated B- and/or T-cell clones. Although isolation of B-cells from synovium without modification of the expression of surface markers is challenging, it is important to commence these analyses in the near future in order to develop more selective immunotherapy.

We wish to note that in our efforts to perform a UMI- standardized reproducible quantitative analysis of the BCR repertoire we based our analysis on mRNA of the heavy chain only. One might argue that different cell types from the B-cell lineage, such as B-cells and plasma cells, express different levels of BCR-heavy mRNA. Literature describes a 5-50 fold difference in the expression of BCR mRNA in plasma

cells and B-cells, obviously dependent on the activation status of the B-cell [24,25]. As noted above, the observed BCR clonality might thus in part reflect selective maturation rather than proliferation in the individual joint. We do not think that the fact that our analysis is only based on heavy chain analysis renders our results less valid: the enormous variability in the heavy chain produced by somatic rearrangement and mutation is sufficient to distinguish the different clones for the purpose of our clonality analysis. However, for further functional assays identification of the linked light chain is clearly desirable, e.g. using single cell analysis.

In conclusion, BCR repertoires in biopsies from inflamed ST from different sites within one joint, from different joints and from SF share dominant BCR clones, while these clones have very low frequencies in PB. This suggests that for some B-cell (receptor) analyses both tissue biopsies and synovial fluid may be equally informative, the latter being more patient-friendly.

Our results show that shared underlying B-cell responses might underly inflammation in different joints. This supports the idea that antigen- and/or receptor-specific therapies targeting a limited set of B-cell and/or T-cell receptor clones in individual patients might be feasible in rheumatoid arthritis or even in the “immune-mediated inflammatory diseases”-group at large.

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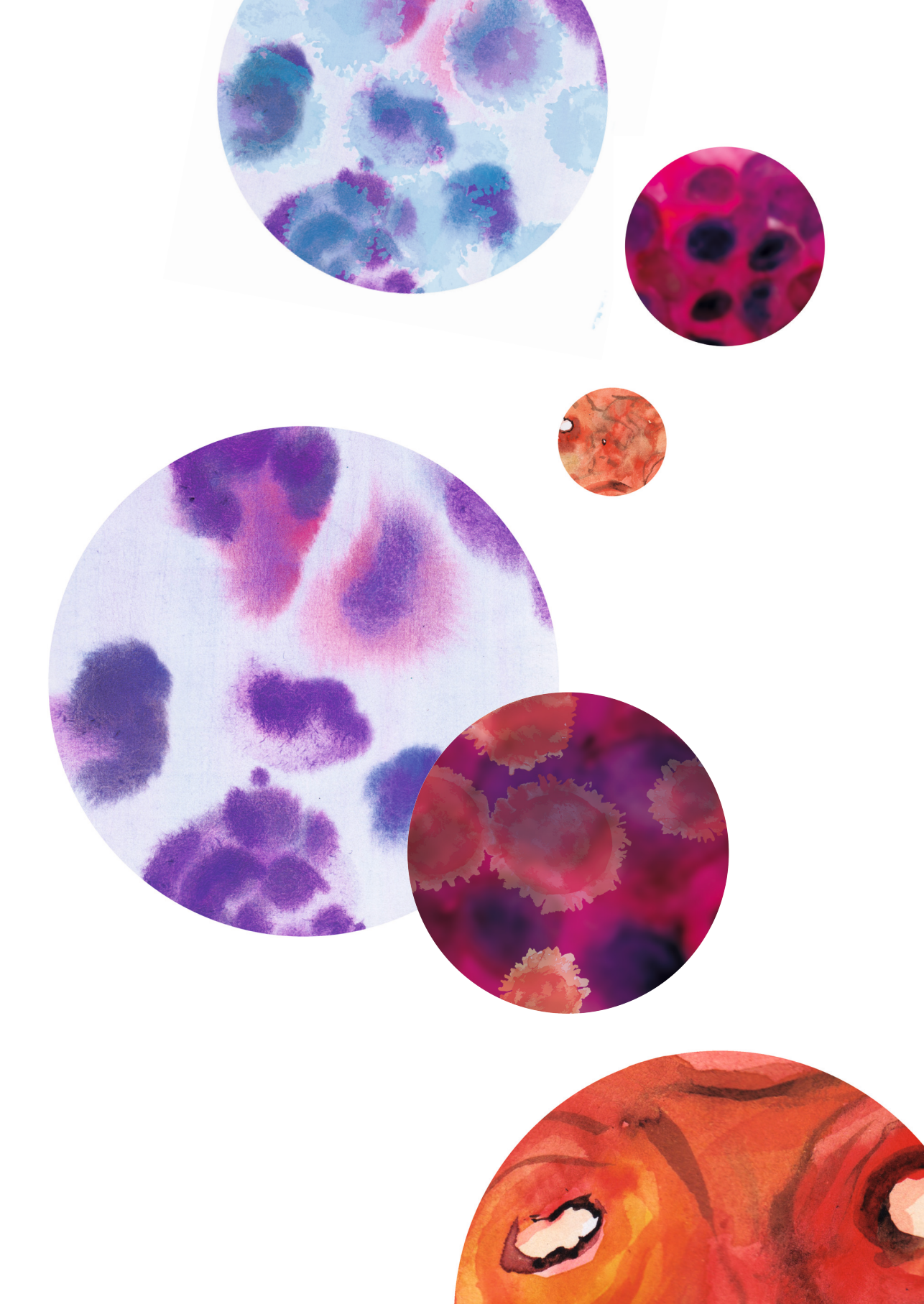
PART II

Treatment options in early stages of rheumatoid arthritis



Prevention of rheumatoid arthritis: A systematic literature review of preventive strategies in at-risk individuals

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4

Abstract

Background Rheumatoid arthritis (RA) is an autoimmune disease characterized by symmetrical peripheral polyarthritis in the hands and/or feet, leading to long-term disability if not treated effectively. RA is preceded by a preclinical phase, in which genetically predisposed individuals accumulate environmental risk factors, and during which autoimmunity develops, followed by the emergence of non-specific signs and symptoms before arthritis becomes manifest. Early treatment in at-risk individuals – i.e. before the disease is fully established - has the theoretical potential to delay or prevent disease onset, with a positive impact on both patients' life and society.

Objectives We aimed to understand the feasibility of preventive treatment in at-risk individuals, taking into account recently performed studies and ongoing clinical trials, as well as patient perspectives.

Methods We performed a systematic literature review (SLR) on Medline and Embase, searching articles published between 2010 and 2021 with the following key-words: "Rheumatoid arthritis", "arthralgia", "pre-treatment" or "prevent".

Results Our SLR identified a total of 1821 articles. Articles were independently screened by two researchers. A total of 14 articles were included after screening, and an additional 8 reports were manually included. We identified ten relevant clinical trials performed in at-risk individuals, or in individuals with undifferentiated inflammatory arthritis. Although no treatment was shown to prevent RA onset, early treatment with rituximab and abatacept delayed onset of full-blown RA, and both conventional and biological disease-modifying anti-rheumatic drugs (DMARDs) decreased disease-related physical limitations and increased DAS28-defined remission, at least temporarily.

Conclusions This SLR demonstrates that early treatment of at-risk individuals may be effective in delaying RA onset, thereby decreasing disease-related limitations in individuals in the pre-clinical phase of RA. Whether this may ultimately lead to prevention of RA remains to be determined.

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease that affects 0.5-1% of the population worldwide [1,2]. The onset can occur at any age, with a mean around 40-50 years. RA is characterized by symmetrical peripheral polyarthritis in the hands and/or feet, usually accompanied by systemic inflammation and other clinical manifestations. In untreated or non-responsive patients the inflammatory process eventually results in joint destruction, in some cases resulting in severe disability [3,4]. RA is also associated with an increased risk of cardiovascular disease and interstitial lung disease [5,6]. RA has a big impact on both the individual and the society. While it is possible for some patients to reach remission, not all patients respond to treatment. In addition to the patients affliction this can result in loss of work productivity and impairment of social activities. Even those who do respond are subjected to life-long treatment with costly therapies.

There are two major subtypes of RA, based on the presence or absence of autoantibodies against specific antigens, most notably IgM-rheumatoid factor (RF) and antibodies against citrullinated proteins (ACPA). In general, ACPA-positive patients have earlier onset of disease (i.e. at younger age) than ACPA-negative patients, and often display a more severe clinical disease [7]. In line with this, ACPA-positive patients also have lower remission rates after initiation of disease-modifying anti-rheumatic drug (DMARD) treatment and more joint damage, although clinical manifestations at diagnosis are often indistinguishable between ACPA-positive and ACPA-negative patients [8].

In the evolution from healthy to full-blown RA different disease phases can be discriminated: an initial at-risk period (with genetic and/or environmental risk factors), a phase of clinically silent autoimmunity (e.g. the presence of ACPA), followed by clinically suspicious arthralgia (CSA), undifferentiated arthritis (UA), early RA, and established RA [9,10]. However, some phases might not be seen in all the patients. Seropositive and seronegative patients have overlapping but distinct risk factors [11]. The preclinical at-risk phase of seropositive individuals is characterized by immune system activation, production of autoantibodies and non-specific musculoskeletal signs and symptoms, mostly arthralgia [12]. Moreover, individuals may experience fatigue, pain, and transient swelling of the joints [13]. Both circulating antibodies and high levels of C-reactive protein (CRP), indicating some level of systemic inflammation, can be found up to five years before RA onset and signify an elevated risk of developing RA [14,15].

Both ACPA-positive and ACPA-negative patients may have a symptomatic stage before developing overt clinical arthritis, which is characterized by the presence of arthralgia and/or subclinical inflammation and other circulating auto-antibodies in the seropositive patients. In the CSA phase, ACPA-positive and ACPA-negative patients have somewhat different clinical manifestations, with fewer tender joints but more rapid progression to RA in ACPA-positive patients [16]. The identification of seronegative at-risk individuals in the pre-clinical stage before the manifestation of arthritis is quite challenging and therefore most trials aimed at prevention of RA focus on seropositive at-risk individuals.

In the time preceding disease manifestation and in the earliest stages of disease, T cells shift towards a pro-inflammatory phenotype, with accumulation of expanded T cells in the synovium and increased levels of serum pro-inflammatory cytokines, including IL-2 [17–20]. B lineage cells are also altered in pre-RA individuals, with high levels of IgA plasmablasts in the peripheral blood [21]. Moreover, patients develop anti-modified protein antibodies (AMPA) against post-translationally modified proteins, such as acetylated and carbamylated proteins, closer to disease onset [22,23]. These findings suggest that the immune system is already derailed years before RA onset, which would potentially allow for targeted interventions to prevent or at least delay disease onset.

It has been shown that early treatment initiation in RA patients improves disease signs and symptoms, with lower disease scores and improved physical functioning as well as reduced radiographic progression [24]. Many patients also experience a rapid decrease in their work ability before treatment initiation and although treatment was unable to reverse disease-associated disability, it stabilized the need for sick leave and prevented incapacity for work [25]. Treatment in the preclinical phase of disease might lead to less complaints in the arthralgia phase, prevention of joint damage and improved work ability.

In order to prevent disease development it is important to identify which patients would benefit the most from a targeted intervention since on average only 30% of seropositive individuals will develop RA, and since many different factors influence disease risk [26,27]. Family history of RA is associated with increased disease risk by three to ten times [28,29]. Genetic predisposition, such as the presence of specific alleles in the HLA-DRB1 locus called shared epitope (SE), and environmental exposure both have a role in disease risk [30]. One of the main disease risk factors in seropositive individuals is smoking which significantly increases the risk of progression towards RA (and this risk may persist for some years after smoking

cessation) especially when paired with SE [10,31,32]. Additional environmental risk factors are an unhealthy diet, obesity, lack of exercise, and stress, while moderate alcohol consumption may be a protective factor [33–36]. The stratification of at-risk individuals based on their risk factors may allow the identification of those patients who might benefit the most from early preventive actions and/or pharmaceutical interventions. Indeed, in one prediction model which used demographic, clinical, and serological data, patients were stratified into three categories based on their risk of developing RA. Individuals in the low-risk group had only 12% probability of developing RA in the next 5 years. This probability increased to 81% in the group with high risk, identifying the patients who might benefit the most from early intervention strategies [37].

At the moment, no formal treatment recommendations exist for individuals in the pre-clinical stage of RA after presenting with arthralgia; patients are monitored, but as a rule they do not receive any DMARD treatment until clinical signs and symptoms of arthritis and/or formal diagnosis of RA is established. The current study was designed to better understand the feasibility of interventional studies in at-risk individuals, taking into consideration both patient perspectives and the results of recently performed or currently ongoing intervention trials. We distinguished true preventive trials, in which at-risk individuals, including those with CSA, who did not experience clinical arthritis yet, were subjected to targeted interventions, from very early treatment strategies in which UA or early RA patients received treatment. We took note of the SLR and meta-analysis by Hilliquin et al. from 2018, which addressed the same but felt that the number of original reports published since then warranted a renewed investigation [38].

Methods

Search Strategy

We performed a systematic literature search on Medline and Embase; we focused on articles published between 2010 and 2021. The keywords we used were the following: “exp Arthritis, Rheumatoid/ or ((rheuma* adj3 (gout or disease* or arthritis)) or RA).ti,ab,kf”; “pretreatment or early or suspect or probable or (preclinical or onset or profylaxis or pre-treatment or arthralgia)).ti,ab”; “(prevent* or remission or progression).ti,ab,kf”.

Study selection criteria

We selected papers that reported randomized controlled trials (RCTs), trial protocols, summary of trials, and patients’ prospective on the topic. The search was limited to

articles published in English between 2010 and 2021. Articles were independently screened by two researchers (GF, AM) based on title and abstract, followed by full text. Selected articles were divided into three categories depending on their subject: background information, RCTs, and patient perspectives. Additional articles, conferences abstracts and reports were manually included after the search by the authors. The search, screening, and articles inclusion were done accordingly to the Cochrane Collaboration guidelines for a systematic literature review on interventions [39].

Summary of results

A total of 1821 articles were screened, following the before mentioned criteria. Of those, 18 duplicates and 122 papers in non-English were removed. Thus, a total of 1681 articles were eligible for inclusion. At the end of the first screening step, 22 articles were included and 8 additional relevant reports were added. Hand-selected reports included published articles and abstract presented at conferences. After the second screening phase, 8 articles were excluded, for a final count of 22 articles. Figure 1 reports a summary of the search and inclusion process.

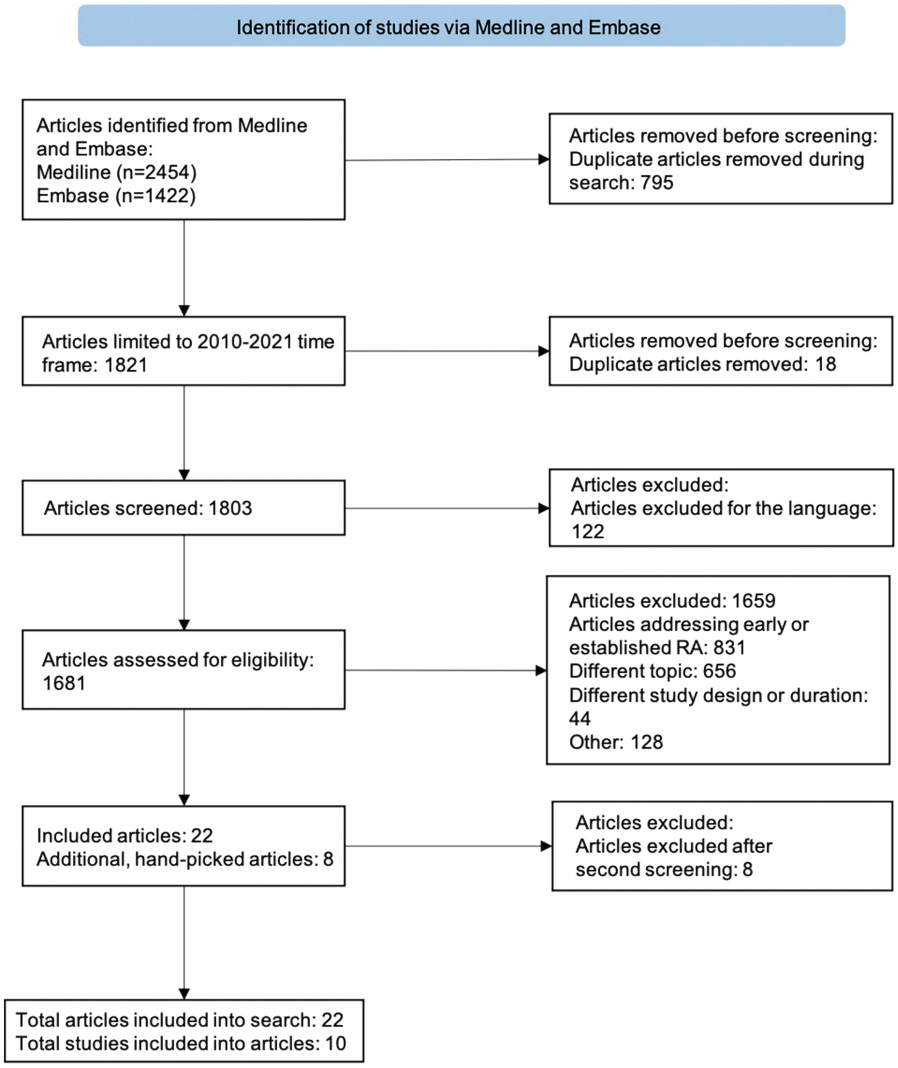

Figure 1 | Flow of articles inclusion
Papers were collected according to the search strategy described in methods, and screened by two independent reviewers (AM and GF).

Results

Retrieved articles

Our search identified a total of 14 articles, which included RCTs, patients' perspectives, and literature overviews of our topic. Six additional articles (five trials and one patients' perspective study), one abstract and results presented at the Advances in Targeted Therapies meeting that took place in Marseille, France in March 2022 were included manually by the authors.

Randomized-controlled trials

In total, we thoroughly reviewed ten RCTs (Table 1):

- one RCT in seropositive first-degree relatives of RA patients, who were treated with conventional synthetic (cs)DMARD (StopRA study),
- six RCTs in arthralgia patients, where participants were treated with glucocorticoids (dexamethasone trial), csDMARDs (TREAT EARLIER study), biological (b) DMARDs (ARIAA trial, APIPPRA trial, PRAIRI trial), and statins (STAPRA study),
- two trials in UA, in which patients were treated with csDMARDs (PROMPT study) and bDMARDs (ADJUST trial),
- one trial in inflammatory arthritis, in which patients were treated with glucocorticoids (SAVE study).

Figure 2 shows the timeline (Figure 2A) of the included trials and their distribution in relation to the different (pre-clinical) phases of RA (Figure 2B).

Table 1 | Summary table of preventive interventional trials in individuals at risk of developing rheumatoid arthritis

Trial name	Inclusion criteria	Number of patients (treatment vs placebo)	Intervention	Time of follow-up	Primary outcome	Secondary outcome	Results	Reference
ADJUST study	- UA - ≤3 criteria for RA - Clinical synovitis - ACPA positivity - Symptoms <18 months	56 patients 26 treatment vs 24 placebo	8 infusions of 10 mg/kg of abatacept (10 mg/kg) for 6 months	- 6 months - 1 year - 2 years	- RA progression as per 1987 ACR criteria at 1 year	- Radiographic assessment of hands/wrists and feet at 6 months, 1 and 2 years - MRI of hand/wrists at 2 weeks, 6 months, and 1 year - ACPA and RF levels at 6 months and 1 year - DAS28 score based on CRP levels - Swollen and tender joints (28-joint count)	- Decreased but not significant RA progression in treatment group (46.2%) vs placebo (66.7%) - Improved osteitis, erosion, and synovitis at 6 and 12 months in treatment vs worsening of scores in placebo - Decreased ACPA positivity at 6 months (90.9%) and 1 years (86.7%) - DAS28-defined remission at 6 months (71.4% vs 35%) and 1 year (47.4% vs 27.4%) - Higher percentage of patients without swollen cells at 6 months (62.5% vs 14%) and 1 year (30% vs 14%)	[57,58]

Table 1 | Summary table of preventive interventional trials in individuals at risk of developing rheumatoid arthritis (continued)

Trial name	Inclusion criteria	Number of patients (treatment vs placebo)	Intervention	Time of follow-up	Primary outcome	Secondary outcome	Results	Reference
APIPPRA study	- CSA - ACPA and RF positivity or high ACPA serum levels	206 patients	125 mg weekly abatacept for 1 year	- 52 weeks - 2 years	- Feasibility, efficacy, and acceptability of treatment - Characterization of immune response - Time to development of clinically apparent synovitis in ≥3 joints or RA per ACR/EULAR 2010 criteria	- RA progression - Disease activity - Percentage of patients requiring DMARDs - Adverse events - Patients' perception	- Study concluded. Results expected in 2022	[62]

Table 1 | Summary table of preventive interventional trials in individuals at risk of developing rheumatoid arthritis (continued)

Trial name	Inclusion criteria	Number of patients (treatment vs placebo)	Intervention	Time of follow-up	Primary outcome	Secondary outcome	Results	Reference
ARIAA study	- Arthralgia - ACPA positivity - Subclinical inflammation (tenosynovitis, synovitis, or osteitis in MRI of the dominant hand)	98 patients (49 treatment vs 49 placebo)	125 mg weekly abatacept for 6 months	- 6 months - 18 months	- Proportion of patients with improvement of synovitis or osteitis with MRI of dominant hand at 6 months	- Synovitis score at 12 and 18 months - Percentage of patients with new arthralgia - Pain score - MRI improvement - Swollen and tender joint - Percentage of patients with RA - DAS28 score	- Decrease in at least one of MRI parameters (tenosynovitis, synovitis or osteitis) at 6 months (62% in treatment vs 31% in placebo group) - Reduced RA progression at 6 months (8.2% vs 34%) and 1 year (35% vs 57%)	[59-61]
Dexamethasone study	- Arthralgia - 2x elevated IgM-RF and/or ACPA levels - HLA-DR SE	83 patients (42 treatment vs 41 placebo)	100 mg of dexamethasone at baseline and 6 months	- 6 months - 5 years	- 50% reduction of autoantibody levels or normalization at 6 months	- RA onset at 5 years (DAS28 or ACR 2010 criteria)	- Reduction of ACPA (22%) and RF (16%) at 1 month - ACPA reduction at 6 months - No difference in RA onset (20% in treatment vs 21% in placebo)	[47-48]

Table 1 | Summary table of preventive interventional trials in individuals at risk of developing rheumatoid arthritis (continued)

Trial name	Inclusion criteria	Number of patients (treatment vs placebo)	Intervention	Time of follow-up	Primary outcome	Secondary outcome	Results	Reference
PRAIRI study	- Arthralgia - ACPA and RF positivity - CRP>0.6 mg/L - Subclinical synovitis (detected either by MRI with gadolinium or ultrasound) - Absence of inflammatory arthritis	81 patients (41 treatment vs 40 placebo)	100 mg methylprednisolone + single gift of 1000 mg rituximab at baseline	- 12 months - 18 months - 5 years	- Time to development of clinical arthritis	- Circulating autoantibody levels - Peripheral T and B cell numbers and subpopulations	- Decreased baseline risk of RA at 12 months (55% in treatment group) and 18 months (53%) - 12 month delay of RA development after rituximab treatment - Significant depletion of B cells - Significant decrease in IgA-RF, IgM-RF and total IgM after treatment	[64]

Table 1 | Summary table of preventive interventional trials in individuals at risk of developing rheumatoid arthritis (continued)

Trial name	Inclusion criteria	Number of patients (treatment vs placebo)	Intervention	Time of follow-up	Primary outcome	Secondary outcome	Results	Reference
PROMPT study	- UA	110 patients (55 treatment vs 55 placebo)	15 mg weekly methotrexate, increased every three months if DAS>2.4	- 3 months - 6 months - 9 months - 12 months - 18 months - 30 months - 5 years	- Development of RA per 1987 ACR criteria	- Radiographic joint damage progression of hand and feet at all time-points except 3 months	- Delay in ACPA-positive, but not ACPA-negative, patients with high risk of RA at 1 year - No significant difference in RA onset and radiographic progression at 5 years	[49-52]
SAVE study	- Inflammatory arthritis <16 weeks	383 patients (198 treatment vs 185 placebo)	120 mg methylprednisolone at baseline	- 12 weeks - 52 weeks	- Clinical remission (no joint swelling, ≤2 tender joints, normalized CRP)	- Additional use of glucocorticoids or DMARDs	- No significant difference in remission (16.2% treatment vs 17.8% placebo) - Now difference in additional glucocorticoids (24.4% *vs* 27.0%) or DMARDs (43.4% *vs* 46.5%)	[46]

Table 1 | Summary table of preventive interventional trials in individuals at risk of developing rheumatoid arthritis (continued)

Trial name	Inclusion criteria	Number of patients (treatment vs placebo)	Intervention	Time of follow-up	Primary outcome	Secondary outcome	Results	Reference
STAPRA study	- Arthralgia - ACPA LN or - ACPA and IgM-RF positivity	62 patients (31 treatment vs 31 placebo)	40 mg atorvastatin daily for 3 years	- 1 month - 6 months - 5 years	- Development of clinical arthritis (≥1 swollen joint out of SJC44)	- Development of RA per ACR/EULAR 2010 criteria	- Prematurely stopped – Treatment: 5-26 months follow-up; Placebo: 3-17 months follow-up - No significant difference in RA development (29% in treatment vs 19% in placebo group)	[68,69]
Stop RA	- FDR - 2x elevated anti-CCP3 levels - Absence of clinical joint inflammation	114 patients	200-400 mg/daily Hydroxychloroquine for 1 year	- 52 weeks - 3 years	- RA progression as per ACR/EULAR 2010 criteria	- Change in disease activity (joint pain, swelling, stiffness, and fatigue) at 1 and 3 years - Changes in self-reported Physical, Mental and Social Health Quality of Life Measures at 1 and 3 years	- Waiting for results (primary completion date set for November 2023)	[40]

Table 1 | Summary table of preventive interventional trials in individuals at risk of developing rheumatoid arthritis (continued)

Trial name	Inclusion criteria	Number of patients (treatment vs placebo)	Intervention	Time of follow-up	Primary outcome	Secondary outcome	Results	Reference
TREAT EARLIER	- CSA less than 1 year - Subclinical inflammation of hand/feet joints by MRI scan of hands or forefeet	236 patients (119 treatment vs 117 placebo)	120 mg methylprednisolon at baseline + increasing dose of methotrexate up to 25 mg/week	- 52 weeks - 2 years	- Clinical arthritis for ≥ 2 weeks - RA progression as per ACR/EULAR 2010 criteria - DMARD-free status	- Productivity and impairment scale - Symptoms (pain, fatigue, morning stiffness)	- No difference in RA onset - Delayed RA onset in subgroup of patients with high risk - Decreased joint inflammation by MRI in treatment group at 1 year -Decreased disease-related symptoms, physical impairment and loss of productivity in treatment group at 1 year	[53-55]

The effectiveness in preventing the development of rheumatoid arthritis in patients with Undifferentiated inflammatory arthritis and to evaluate Safety and Tolerability. UA=Undifferentiated arthritis. RA=Rheumatoid arthritis. ACPA=Anti-Citrullinated Proteins Antibodies. ACR=American College of Rheumatology. MRI=Magnetic Resonance Imaging. RF=Rheumatoid Factor. DAS28=Disease Activity Score of 28 joints. CRP=C-reactive protein. APIPPRA=Arthritis Prevention In the Pre-clinical Phase of RA with Abatacept. CSA=Clinical Suspected Arthralgia. EULAR= European Alliance of Associations for Rheumatology. DMARDs=Disease Modifying Anti-Rheumatic Drugs. ARIAA=Abatacept Reversing subclinical Inflammation as measured by MRI in ACPA positive Arthralgia. HLA= Human Leukocyte Antigen. SE=Shared Epitope. PRAIRI=Prevention of clinically manifest rheumatoid arthritis by B-cell directed therapy in the earliest phase of the disease. PROMPT=PRObable rheumatoid arthritis: Methotrexate versus Placebo Treatment. SAVE=Stop Arthritis Very Early. STAPRA=STAtins to Prevent Rheumatoid Arthritis. SJC44=Swollen Joint Count among 44 joints. TREAT EARLIER=TREAT Early Arthralgia to Reverse or Limit Impending Exacerbation to Rheumatoid arthritis.

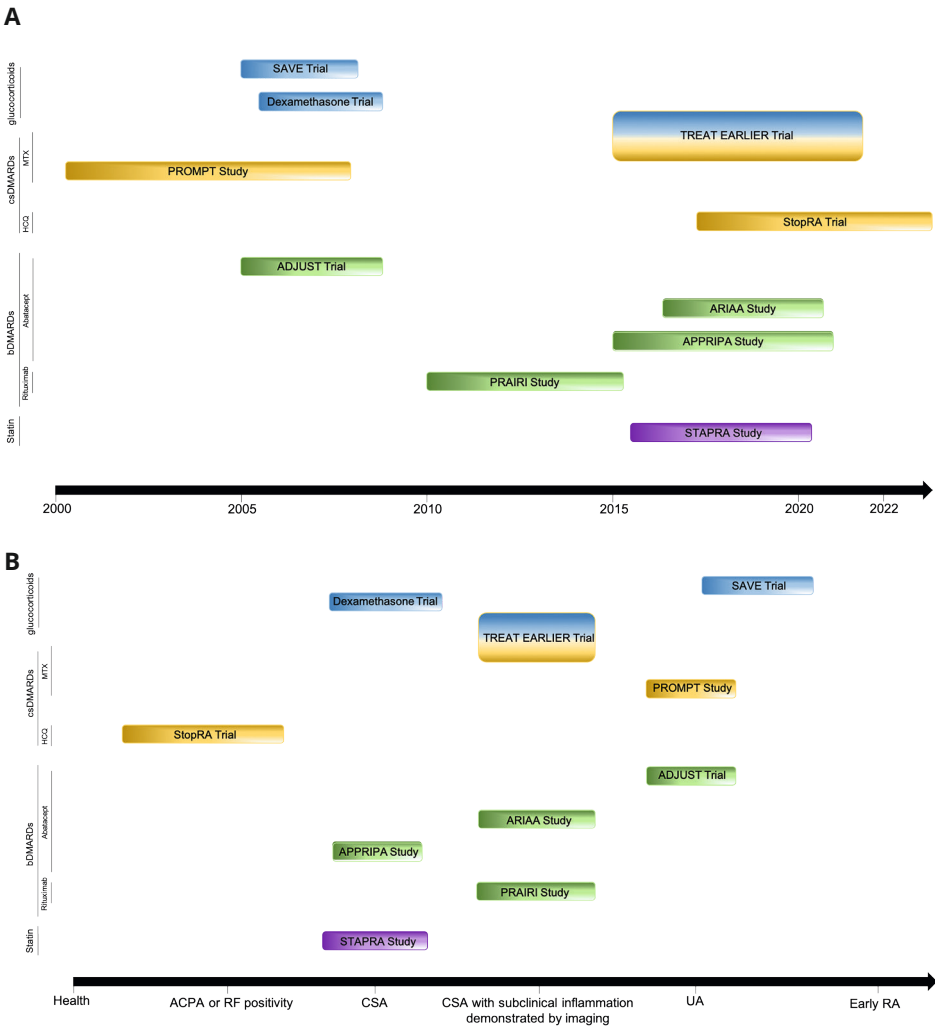

Figure 2 | Overview of preventive interventional trials
In blue trials investigating glucocorticoids, in yellow conventional synthetic (cs) disease-modifying anti-rheumatic drugs (DMARDs), in green biological (b)DMARDs, and in purple statins. HCQ=hydroxychloroquine. ADJUST=Abatacept study to Determine the effectiveness in preventing the development of rheumatoid arthritis in patients with Undifferentiated inflammatory arthritis and to evaluate Safety and Tolerability. APIPPRA=Arthritis Prevention In the Pre-clinical Phase of RA with Abatacept. ARIAA=Abatacept Reversing subclinical Inflammation as measured by MRI in ACPA positive Arthralgia. PRAIRI=Prevention of clinically manifest rheumatoid arthritis by B-cell directed therapy in the earliest phase of the disease. PROMPT=PRObable rheumatoid arthritis: Methotrexate versus Placebo Treatment. SAVE=Stop Arthritis Very Early. STAPRA=STAtins to Prevent Rheumatoid Arthritis. TREAT EARLIER=TREAT Early Arthralgia to Reverse or Limit Impending Exacerbation to Rheumatoid arthritis. (A) Timeline of preventive interventional studies. (B) Overview of preventive interventional trials based on their target population.

Glucocorticoids

Glucocorticoid are immunosuppressive and anti-inflammatory drugs which inhibit leukocyte migration to the site of inflammation and production of pro-inflammatory cytokines and soluble factors [40]. Glucocorticoids are used as first line medication in the treatment of RA, especially as bridging and/or local therapy in combination with cDMARDs [41,42]. They are also frequently used during flares, given their rapid immunosuppressive action [43]. Side effects are dose- and time-dependent, and accordingly short-term and/or low dose administration of glucocorticoids (≤ 5 mg/day) is associated with a lower risk of side-effects [43,44]. Therefore, the European Alliance of Associations for Rheumatology (EULAR) recommendations for the use of glucocorticoids advocate tapering as soon as possible [41]. In case of long-term use, a low dose (≤ 5 mg/day) is preferred [45].

The Stop Arthritis Very Early (SAVE) trial investigated the efficacy of glucocorticoids in patients with early inflammatory arthritis of >1 joint. Patients were included if symptom duration was less than 16 weeks, and based on the number of swollen joints stratified into either monoarthritis or oligoarthritis (≤ 3 swollen joints) or polyarthritis (> 3 swollen joints). Subsequently, patients were treated with either a single intramuscular injection of 120 mg methylprednisolone or placebo (NaCl 0.9%). Paracetamol and/or non-steroidal anti-inflammatory drugs (NSAIDs) could be taken on demand. Patients were followed up for one year and the study's primary outcome was the presence of clinical remission at 12 weeks and at 52 weeks after injection, defined as the absence of joint swelling, not more than two tender joints and normalized CRP without additional glucocorticoid or DMARD treatment. A total of 383 individuals were included in the study and randomized to treatment (198 patients, 51.7%) vs. placebo (185 patients, 48.3%). No significant difference was found in the percentage of patients who reached remission in the two groups (16.2% in the treatment group vs. 17.8% in the placebo group). Moreover, no difference was found in the percentage of patients who required additional glucocorticoids (24.4% vs. 27.0%, respectively) or DMARDs (43.4% vs. 46.5%, respectively) in the two groups. Patients with monoarthritis had a statistically higher percentage of remission (27.7%) and lower symptom duration than patients with polyarthritis (10.2%), while no significant difference was found between the treatment groups [46].

The dexamethasone trial by Bos et al. recruited patients with arthralgia who had at least a two-fold increased level of IgM-RF and/or ACPA measured at least twice with an interval of more than 4 weeks. Patients were only included if positive for HLA-DR SE and were excluded in case of active hepatitis C and/or EBV infection,

corticosteroid use or contraindications for their use, and co-morbidities that would decrease patients' life span. After inclusion, patients were randomized to receive 1-2 intramuscular injections (depending on the response to the first injection) of 100 mg of dexamethasone or placebo at baseline and at 6 weeks [47]. Eighty-three patients were recruited and randomized to active treatment (n=42) vs. placebo (n=41). The primary endpoint was a 50% reduction of antibodies or normalization after 6 months of treatment. The secondary outcome of this trial was RA development 5 years after treatment, based on the Disease Activity Score of 28 joints (DAS28) and the fulfilment of ACR criteria for classification. Although the primary endpoint was not reached (in each group only one patient reached the primary endpoint), the study did demonstrate a substantial reduction of ACPA (22% reduction in treatment group vs. 3% increase in placebo group) and, to a lesser extent, IgM-RF (14% reduction in treatment group vs. 1% increase in placebo group) after 1 month. ACPA reduction persisted for up to six months (13% reduction in treatment group vs. 12% increase in placebo group at 3 months, 8% reduction in treatment group vs. 2% increase in placebo group at 6 months), and IgM-RF reduction persisted up to three months (6% reduction in treatment group vs. 6% increase in placebo group), but neither ACPA nor IgM-RF reduction was associated with prevention or delayed onset of RA. The median follow-up time was 26 months, and no significant difference in RA development was observed in the two groups (20% in dexamethasone group, 21% in placebo group). Also, no significant difference in the median DAS28 score was found between the treatment (2.9; IQR 2.4-4.2) and placebo (3.7, IQR 3.2-4.1) groups [48].

csDMARDs

Methotrexate

Methotrexate has been the cornerstone of RA treatment for decades [41] and rheumatologists have ample experience with this drug, which has a favorable efficacy and safety profile. Consequently, it is not surprising that interventions with this csDMARD have been performed in the early and preclinical phases of the disease as well.

The PRObable rheumatoid arthritis: Methotrexate versus Placebo Treatment (PROMPT) study was not a true prevention trial as it evaluated the efficacy of methotrexate treatment in patients with UA, regardless of their ACPA status. Patients had symptoms for less than 2 years and were also DMARD-free [49]. The patients (110 patients in total; 55 patients in each arm) were randomized to either receive active treatment or placebo regardless of their specific risk for developing RA. Initial treatment was with methotrexate (15 mg/week) and the dosage was increased

every three months if DAS was >2.4. After 1 year, treatment was discontinued and patients were followed for 60 months. The primary outcomes were progression to RA, defined by the 1987 ACR criteria, and radiographic joint damage of the hand and feet joints [50]. After trial conclusion, a risk stratification score was applied to the results based on a validated prediction model, and patients were stratified based on their risk [51,52]. Only patients with a high risk of developing RA were considered in the analysis after stratification [52]. The PROMPT study found that methotrexate treatment delayed RA onset in ACPA-positive, but not in ACPA-negative patients. Without risk stratification, no difference was found in the number of patients who developed RA in the treatment arm compared to placebo [50]. Upon risk stratification and selection of the patients with highest risk of developing RA (11 patients in each arm), a 1-year course of methotrexate reduced the absolute risk of developing RA by 45%, with more frequent drug-free remission after 5 years compared to placebo [52]. However, there was no significant lasting effect of methotrexate at 5 years after treatment [49].

The TREAT Early Arthralgia to Reverse or Limit Impending Exacerbation to Rheumatoid arthritis (TREAT EARLIER) study recruited patients with CSA and arthralgia for less than 1 year coupled with subclinical inflammation of the small joints of either hands or feet, as identified by unilateral MRI findings. Patients were treated with a single intramuscular dose of 120 mg methylprednisolone followed by methotrexate in increasing dosages up to 25 mg/week for 1 year, versus placebo for both, in a double-blinded manner. In the last 4 weeks of treatment, methotrexate was gradually reduced before being stopped at week 53. Patient recruitment was independent of their serological status (i.e. both RF/ACPA positive and negative individuals were included as long as they were at risk) and they were followed for an additional year. The primary endpoint of the study was either development of clinical arthritis that lasted >2 weeks or RA by the American College of Rheumatology (ACR)/ EULAR 2010 RA classification criteria. The study's co-primary endpoint was DMARD-free status for two years, and secondary endpoint was patient functioning, assessed by Health Assessment Questionnaire disability index (HAQ-DI) and Work Productivity And Impairment (WPAI), as well as symptoms (pain, fatigue, morning stiffness) at baseline and regular 4 month intervals after treatment initiation [53,54]. Joint inflammation was explored using MRI at baseline and 4, 12, 24 months after treatment initiation. A total of 236 participants (119 participants in the treatment group, 117 in the placebo group) were included in the study; three participants were lost to follow-up in the treatment group, while 5 participants were lost in the placebo group. Treatment with methotrexate did not prevent RA onset (20 % of RA onset in treatment group vs. 18% in placebo group), although decreased joint inflammation was detected by

MRI during treatment and up to a year after treatment was stopped (mean difference of -1.4 points). Moreover, patients treated with methotrexate had reduced disease-related symptoms (pain=-8 on 1-100 scale in treatment vs- placebo, morning stiffness=-12, and functional impairment), less physical limitations and less loss of work productivity, regardless of their autoantibody status. High risk participants, who had a risk of developing RA higher than 70% at baseline, also had a delay in RA onset during treatment, although disease frequency was similar at 2 years after treatment initiation (67% in both groups) [54,55]. Interestingly, most beneficial effects occurred in the first 4 months and were sustained throughout the rest of the study despite a moderate target dose treatment adherence of 53% at 52 weeks in the methotrexate group.

Hydroxychloroquine

Hydroxychloroquine is a weak basic that has been used for centauries as antimalarial drug. Hydroxycloroquine accumulates in the lysosome, interfering with its activity and autophagy, preventing immune cell activation and cytokine production. On T cells, hydroxychloroquine also causes reduction of CD154 expression [56].

The StopRA study is currently being conducted and focuses on first degree relatives of RA patients with serum levels of anti-cyclic citrullinated peptide (anti-CCP3) two or more times higher than normal, but without clinical joint inflammation. The study aims to recruit 114 participants, who are randomized to either receive 200-400 mg/daily hydroxychloroquine (based on ideal body weight) or placebo, for 12 months and followed for 2 additional years. The primary endpoint is the number of patients who progress to clinically apparent RA, defined by ACR/EULAR 2010 classification criteria. Secondary outcomes include changes in disease activity defined by joint pain, swelling, stiffness, and fatigue at 12 and 36 months, as well as self-reported Physical, Mental and Social Health Quality of Life Measures. Results should be available after the primary completion date by the end of 2022 [40].

Biological DMARDs

Abatacept

Abatacept is a human fusion protein of CTLA-4 and the Fc portion of IgG that targets CD80 and CD86 on antigen presenting cells, thereby preventing binding to CD28 expressed on T cells and subsequent activation of these cells via downstream signaling pathways [57].

The Abatacept study to Determine the effectiveness in preventing the development of rheumatoid arthritis in patients with Undifferentiated inflammatory arthritis and to evaluate Safety and Tolerability (ADJUST) trial assessed the efficacy of abatacept in preventing RA in patients with UA/very early RA. Patients were considered eligible if they met a maximum of three criteria for RA diagnosis, as classified by ACR 1987 criteria, with concomitant symptomatic clinical synovitis. Moreover, all participants had circulating ACPA and symptoms for less than 18 months. Corticosteroids were allowed with a dose ≤ 10 mg prednisolone or equivalent, but no previous treatment with biological drugs or DMARD was allowed. Fifty-six patients were included in the study and stratified for the presence of erosions. Patients received intravenous abatacept (10 mg/kg) or placebo for six months (days 1, 15, 29, 57, 85, 113, 141, and 169), and were followed-up for 18 additional months [58]. The primary endpoint was RA diagnosis as defined by the 1987 ACR criteria. Additionally, radiographic progression (hands, wrists and feet), bone erosions, osteitis and synovitis (MRI of hand and wrist with the most prominent clinical synovitis), antibody levels, and DAS28 based on CRP levels were assessed. The percentage of patients in the abatacept group who developed RA at 1 year (46.2%) was numerically lower compared to placebo (66.7%) but the difference was not statistically significant. Moreover, abatacept treatment slowed radiographic progression and was associated with improved osteitis, erosion, and synovitis scores at 6 months and 1 year, while patients in the placebo group had worsening of their scores. ACPA positivity was reduced at 6 months (90.9%) and 1 year (86.7%) in the abatacept group, but remained unaltered in the placebo group. Abatacept also caused a decrease in the percentage of RF-positive patients at 6 months (59.1%) and 1 year (73.3%) in comparison with baseline (85.7%), which was not observed in the control group. Moreover, 71.4% of patients treated with abatacept had DAS28-defined remission at 6 months vs. 35.0% of placebo treated patients. This was also seen at 1 year (47.4% in abatacept group vs. 28.5% in the placebo group). Of the abatacept patients, 62.5% had zero swollen and tender joint after 6 months, which decreased to 30.0% at 1 year follow-up, whereas the proportion of patients in the placebo group with zero swollen and tender joints remained constant at 6 and 12 months (14.3%) [57].

The Abatacept Reversing subclinical Inflammation as measured by MRI in ACPA positive Arthralgia (ARIAA) study investigated the efficacy of abatacept in improving subclinical inflammation in ACPA-positive arthralgia patients as measured by the presence of tenosynovitis, synovitis, or osteitis on MRI of the dominant hand. Patients were randomized to receive either abatacept subcutaneously (125 mg weekly) or placebo (NaCl 0.9%) for 6 months, and were followed for an additional 12 months [59]. Ninety-eight patients received either treatment or placebo, and the

study found a significant reduction in at least one MRI parameter (tenosynovitis, synovitis or osteitis) at 6 months in the treatment arm (62% in the abatacept group vs. 31% in the placebo group), indicating improvement in subclinical arthritis. Moreover, at 6 months, fewer patients in the abatacept arm (8.2%; 4 patients) developed RA compared to the placebo arm (34.7%; 17 patients) [60]. Reduced RA development in the treatment arm was still significant one year after stopping treatment (35% in the abatacept group vs. 57% in the placebo group) [61].

The Arthritis Prevention In the Pre-clinical Phase of RA with Abatacept (APIPPRA) trial is a similar study in patients with CSA and presence of both ACPA and RF or with high serum levels of ACPA alone (i.e. three times higher than the upper limit than normal (ULN)). Exclusion criteria include clinical evidence of joint swelling, previous diagnosis of autoimmune disease, use of corticosteroids within 12 weeks before inclusion used to treat musculoskeletal symptoms and current use of cs/bDMARDs. The study aims to recruit 206 patients who are randomized to either receive weekly subcutaneous injections of 125 mg abatacept for a year or placebo. Patients are followed up for an additional year after the end of treatment. The primary study endpoints are feasibility, efficacy, and acceptability of treatment, and the characterization of immune and inflammatory responses associated with ACPA for the duration of the therapy. Co-primary endpoints are the time to development of clinically apparent synovitis in at least three joints or time to development of RA as defined by the ACR/EULAR 2010 classification criteria. The secondary endpoints of the study are development of RA and assessment of disease activity, the percentage of patients requiring DMARDs, treatment adverse events, and patients' perceptions. Results are expected to be presented by the end of 2022 [62].

Rituximab

Rituximab is a chimeric monoclonal antibody that specifically targets CD20 on B cells, inducing cell death through complement-dependent cytotoxicity and antibody-dependent cytotoxicity [63].

In the Prevention of clinically manifest rheumatoid arthritis by B-cell directed therapy in the earliest phase of the disease (PRAIRI; "Prevention of RA by Rituximab") study, patients with arthralgia without clinical arthritis were treated with rituximab, causing B cell depletion. Patients had to be positive for both ACPA and IgM-RF, but could not have experienced inflammatory arthritis nor received DMARDs in the past. Additional inclusion criteria were detectable CRP at screening with levels higher than 0.6 mg/L (the lower level of detection of high sensitivity CRP assay) or subclinical synovitis as detected either by ultrasound or MRI with gadolinium.

Eighty-one patients were recruited in the study and randomized to receive 100 mg methylprednisolone followed by either a single infusion of 1000 mg rituximab (41 patients) or placebo (NaCl 0.9%; 40 patients). Patients were stratified for age (< 40 years old, or ≥ 40 years old) and sex before randomization, with a scheduled follow up of five years after the intervention. The primary outcome of the study was the time to development of clinical arthritis, defined as swollen and tender joints as observed by two independent investigators. Moreover, the study investigated circulating autoantibody levels and peripheral B and T cell numbers and subpopulation composition through fluorescence-activated cell sorting (FACS) analysis. Rituximab treatment was associated with a reduction of the baseline risk of developing RA at 12 months (55% reduction of risk compared to placebo group) and at 18 months (53% reduction of risk compared to placebo). Moreover, patients treated with a single infusion of rituximab had a delay of RA development of 12 months at the point when 25% of the subjects had developed arthritis ("25th percentile"). In contrast, at the end of follow-up time, the risk of RA development was not statistically significant different between treatment and placebo (34% after a median of 16.5 months vs. 40% after a median of 11.5 months, respectively). In summary, the PRAIRI study demonstrated that depletion of B cells in individuals with arthralgia significantly delayed RA onset, confirming that B lineage cells play an important role in disease pathogenesis, including progression to clinical overt RA [64].

Other strategies

Statins

Statins are lipid-lowering agents with a good safety profile and anti-inflammatory properties: in RA patients, statins improved disease activity scores and caused decreased inflammatory parameters [65]. Additionally, in mice models of RA, statins had a protective effect against disease development, and on a population level, statin use is associated with a reduced risk of developing RA [66,67]. Hence, statins were postulated to have a possible beneficial effect in preventing RA [68].

The STAtins to Prevent Rheumatoid Arthritis (STAPRA) trial investigated the ability of a statin to prevent development of RA in high risk individuals with arthralgia. Patients were required to have either high levels of ACPA (three times the ULN) or both ACPA and IgM-RF positivity without (a history of) arthritis, and were randomized to receive either 40 mg of atorvastatin or placebo daily for 3 years [69]. The primary outcome of the study was development of clinical arthritis, defined as one or more swollen joints out of 44 joints assessed (SJC44). A secondary outcome was the development of RA as defined by the ACR/EULAR 2010 classification criteria. The

study was prematurely stopped due to recruitment difficulties, with a total of 62 patients out of 220 originally planned. 36% of patients in the placebo arm and 19% of patients in the atorvastatin arm dropped out of the study, mainly due to (perceived) side effects. The most common side effect in both groups was muscle pains or cramps (37%). The median duration of treatment was 8 months (5-26 months in the atorvastatin group, 3-17 months in the placebo group) and median follow-up time was also similar between the two groups (17 (IQR 6-28) months for atorvastatin, 14 (IQR 5-36) months for placebo). No significant difference was found in the percentage of patients who developed arthritis (29% in the atorvastatin group vs. 19% in the placebo group) or fulfilled the 2010 ACR/EULAR classification criteria for RA (26% in the atorvastatin vs. 19% in the placebo group). With the limited number of participants, the study was underpowered and formally inconclusive, but the fact that more patients developed RA in the intervention group makes it very unlikely that a true preventive effect exists [68].

Individual perspectives of at-risk individuals on early and preventive treatment

Studies on patient preferences with respect to clinical trials have also investigated the likelihood of patients to participate in preventive studies, as well as what requirements are necessary for participation. Patients with a lower risk of developing RA were less inclined to partake in preventive trials than patients with CSA [70]. Moreover, a ≥ 20% decrease of RA risk was associated with increased willingness to undergo treatment when the probability of serious adverse events was ≤ 10% [71]. Similarly, van Boheemen et al. showed that the willingness to use preventive medications increased from 53% to 69% in at-risk individuals when the disease risk increased from 30% to 70% risk [72]. This clearly indicates that patients require a better understanding of their own risk in order to make decisions regarding preventive treatment. Importantly, this requires personalized risk communication that needs to be understandable for the patient, tailored to their social status and education [70,73]. While effectiveness of treatment has a positive impact on patients' willingness to take medication, perceived risk of side-effects has a negative impact: patients are less likely to participate if the achieved benefit is lower than the perceived expected benefit. Moreover, patients prefer and are more likely to adhere to lifestyle modifications than pharmacological therapies, and have a positive perception of interventions that might lead to substantial decrease or even complete elimination of disease risk. Additionally, treatment needs to be safe and a non-invasive route of administration (i.e. oral tablet taken at home) was usually preferred,

to the point that this aspect might influence patients' perspective on preventive treatment more than the efficacy of the intervention in preventing RA [72,74,75].

Discussion and future perspectives

Preventive strategies focus on the prevention or delay of disease onset. While the concept of preventive therapies is not novel, no clear strategy has yet emerged for the prevention of RA. We systematically reviewed the literature for interventional studies in individuals with either high-risk of RA, UA or very early RA. In the past years, several clinical trials have investigated the safety and effectiveness of early (preventive) treatment in RA, focusing on individuals who were at high risk for disease without a clinical diagnosis (true prevention) and patients with very early disease (UA or early RA). These trials investigated a wide range of drugs, such as glucocorticoids, cs/bDMARDs and statins, some of which are used in clinical practice for disease management. Several of these trials showed that early treatment with csDMARDs and bDMARDs may transiently increase the likelihood of drug-free remission and reduce physical limitations in both arthralgia and UA or very early RA patients. Thus, while glucocorticoids did not have a significant effect on disease prevention, studies focusing on csDMARDs or bDMARDs were able to demonstrate some efficacy of treatment.

An important aspect of preventive trials is their feasibility, both in terms of safety, efficacy, and willingness of patients to participate. At-risk individuals are less likely to participate in trials and to adhere to treatment when largely asymptomatic. Both the STAPRA study and the PRAIRI trial faced difficulties in patient recruitment, which led to early interruption of the study (STAPRA, only 62 participants included out of 175 found eligible), or a reduction in sample size (PRAIRI, 81 participants out of 90 patients planned, due to slow inclusion rate) [64,68]. This reduction in the number of participants poses a serious problem, because it may lead to loss of statistical significance (type 2 statistical error). Tailored educational strategies might be required to improve individuals' awareness and their predisposition towards preventive studies as patients are more inclined to participate when well-informed and if the risk of RA is strongly reduced by an intervention with limited side effects. The involvement of general practitioner might help increase inclusion rates, since they could approach patients in the earliest stages of risk, even before arthralgia or UA manifest. Moreover, the establishment of a registry of at-risk individuals, such as the one created by the Rheuma Tolerance for Cure (RTCure) consortium, could help in the identification and inclusion of more patients in RCTs [76].

It is clear that patients in the earliest stages of disease benefit the most from preventive strategies, and are more likely to have a delay or reduced risk of disease progression upon treatment, as demonstrated by the ARIAA and the PRAIRI study [77]. This points out that early identification of at-risk individuals is of paramount importance. This naturally leads to the need to define specific stratification criteria to select patients, as well as a redefinition of primary study outcomes. In addition, to delaying the onset or preventing RA, a secondary goal of therapy could be to improve quality of life in the preclinical phase of the disease, as was observed in the TREAT-EARLIER trial, and/or to positively alter the disease course if the patient does ultimately develop RA; for example, such a treatment might increase the likelihood to achieve DMARD-free remission or improve the health-related quality of life. Interestingly, in several trials (PRAIRI, ARIAA, and TREAT-EARLIER) certain beneficial effects persisted for ≥6 months after treatment cessation pointing towards the fact that control of inflammation very early in the disease course can result in long-lasting improvement of signs and symptoms.

Of note, a good balance between under- and overtreatment is essential when developing preventive strategies in at risk individuals who are strictly speaking not yet patients, since they have not manifested clinical arthritis [78].

Furthermore, the type of intervention that is effective may also differ across the various preclinical phases of the disease. In the coming years research efforts should be steered towards better understanding the immune alterations in the preclinical phase of RA, for instance by investigating various compartments of the immune system, i.e. not only peripheral blood but also lymph nodes and the bone marrow. Early targeted intervention could result in beneficial effects larger than the effects of treatment initiated at the time of diagnosis (i.e. prevention of structural damage, reduced disability, higher (drug-free) remission rates, etc.). Numerous components of disease pathogenesis could serve as potential prevention targets, including autoantibody generation, inflammation and the immune response. Possible future treatment strategies are the delivery of tolerogenic therapies through nanoparticles, cellular therapies with tolerogenic antigen-presenting cells (i.e. dendritic cells), regulatory T cells and mesenchymal stem cells, immune checkpoint receptor agonists (i.e. PD-1, CD200R or BTLA stimulation) and co-stimulatory molecule blockade, as well as depletion strategies for (subsets of) T and B cells [79].

In summary, several preventive strategies have been shown to delay RA onset when applied in the early stages of the disease and to decrease disease burden at least temporarily, while true prevention has hitherto not (yet) been achieved. Results

from ongoing studies are expected to support and increase our knowledge of disease prevention as well as advance our understanding of the pathophysiological mechanisms underlying RA. Strategies for patient identification and stratification, as well as tailored education and treatment, are also necessary to ultimately reach the goal of disease prevention.

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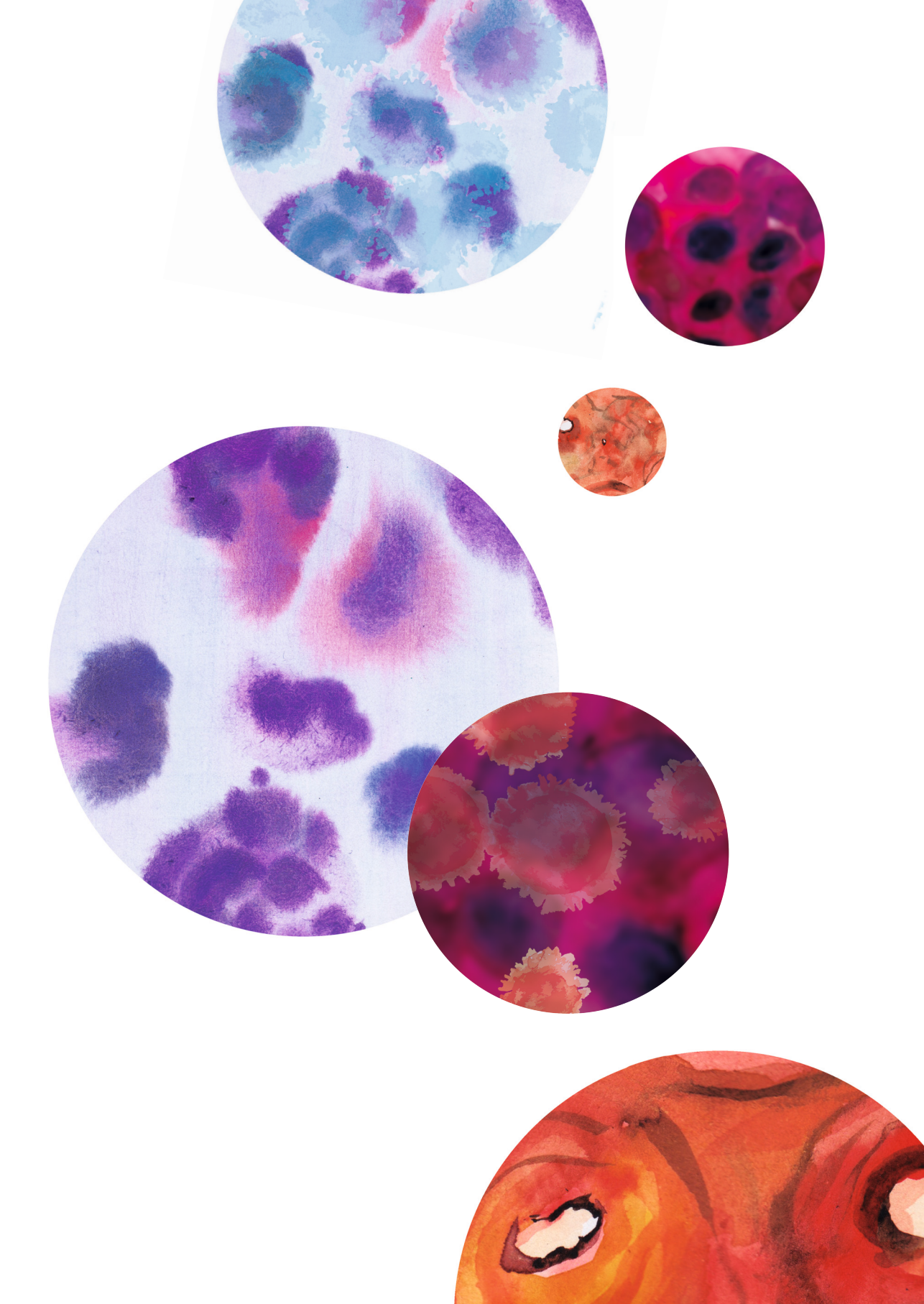
PART III

Dynamics in the of B-cell repertoire after B-cell depletion in different phases of RA



Sensitive B-cell receptor repertoire analysis shows repopulation correlates with clinical response to rituximab in rheumatoid arthritis

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Manuscript submitted

5

Abstract

Background Although B-cell depleting therapy in rheumatoid arthritis (RA) is clearly effective, response is variable and does not correlate with B cell depletion itself.

Methods The B-cell receptor (BCR) repertoire was prospectively analyzed in peripheral blood samples of twenty-eight RA patients undergoing rituximab therapy. Timepoints of achieved depletion and repopulation were defined based on the percentage of unmutated BCRs in the repertoire. The predictive value of early depletion (within one-month post-treatment) and early repopulation (within 6 months post-treatment) on clinical response was assessed.

Results We observed changes in the peripheral blood BCR repertoire after rituximab treatment, i.e., increased clonal expansion, decreased clonal diversification and increased mutation load which persisted up to 12 months after treatment, but started to revert at month 6. Early B-cell depletion was not associated with early clinical response but late depleters did show early response. Patients with early B-cell repopulation with unmutated BCRs showed a significant decrease in disease activity in the interval 6 to 12 months. Development of anti-drug antibodies non-significantly correlated with more B-cell repopulation.

Conclusion Our findings indicate that rather than depletion it is repopulation with unmutated, possibly naïve B cells which induces remission. This suggests that (pre-existing) differences in B-cell turnover between patients explain the interindividual differences in early clinical effect.

Background

The introduction of the B-cell depleting agent rituximab constituted a major revolution in the treatment of autoimmune diseases, which renewed interest in the role of B cells in autoimmunity. In Rheumatoid Arthritis (RA), an autoimmune disease that affects the peripheral joints, pathological studies show a prominent role for B cells in at least a subset of patients. A larger influx of B cells in the synovial tissue has been associated with autoantibody positivity, but also with more radiographic disease progression [1]. This suggests that treatment efficacy might be tightly linked to the contribution of the B-cell compartment in disease pathogenesis.

Although rituximab is clearly effective in certain autoimmune diseases, on the individual level clinical response may vary and is difficult to predict. One of the factors thought to contribute to this variability is the timing and depth of B cell depletion, which has been shown to be very patient specific [2,3]. However, the extent of B-cell depletion does not correlate evidently with clinical response [4]. This might be due to the fact that our tools are relatively insensitive in monitoring B-cell levels in depleted patients, thus detecting repopulation of B cells too late to prevent disease relapse. And in fact, earlier studies confirmed that in rituximab-treated patients high sensitivity techniques are needed to successfully detect B cell signals when conventional flow cytometry fails [2,3]. Hence, there is a clear need for a more sensitive, quantitative diagnostic tool that is able to spot B-cell repopulation very early. If indeed this shows a link with disease progression, this might guide clinicians to adapt therapy accordingly.

In a new prospective cohort of RA patients undergoing rituximab therapy, we used adaptive immune receptor repertoire (AIRR) sequencing to analyze B-cell depletion and repopulation dynamics at the clonal level. We confirmed previously reported effects of rituximab treatment on the peripheral blood B-cell receptor (BCR) repertoire. Furthermore, using BCR repertoire analysis we were able to find a link between B-cell repopulation and clinical efficacy, thus shedding more light on the mechanism behind rituximab efficacy in RA.

Methods

Patients and Samples

Thirty-one patients previously diagnosed with RA according to the 2010 ACR/EULAR criteria, who were about to start with rituximab treatment were included in the ABIRISK consortium multicentric clinical study (NCT02116504) whose primary ob-

jective was to assess predictive factors of anti-drug antibodies (ADA) development (www.abirisk.eu/) [5,6]. The treatment protocol consisted of two intravenous injections of 1000 mg rituximab (Roche, Woerden, The Netherlands) 15 days apart. A second cycle of treatment was allowed after 6 months at the treating physician's discretion (n = 6). Concomitant medications allowed for RA treatment were Disease-modifying antirheumatic drugs (DMARDs), Non-steroidal anti-inflammatory drugs (NSAID) and corticosteroids. No other biologicals were allowed. Patient visits were at baseline and at one, three, six and twelve months after treatment for sample collection and assessment of disease activity using the Disease Activity Score 28 joints (DAS28) based on CRP, or ESR when CRP was not available. Clinical response was assessed using EULAR response criteria [7].

Peripheral blood for BCR repertoire analysis was collected using PAXGene Blood RNA tubes (PreAnalytiX, Breda, The Netherlands) and stored at -80°C. Serum for Anti-Drug Antibodies (ADA) testing was collected in BD SST vacutainers, left to coagulate for at least 30 min, centrifuged at 1,500 g for 10 min at 4°C and then stored at -20°C.

The study protocol received ethical approval in all patient recruiting centers and was performed according to the Declaration of Helsinki. All patients gave written informed consent before participation.

Next-generation sequencing of the B-cell receptor repertoire

RNA extraction was performed using PAXgene isolation kit (Qiagen) according to manufacturer's instructions. Amplification of the B-cell receptor repertoire was performed as previously described and reported in online supplementary figure S1A [8,9]. In case no amplification product was obtained, the amplification was repeated with the addition of carrier RNA from the non BCR-expressing cell line HEK939T. This addition did not alter the sample's BCR repertoire (online supplementary figure S1B-C).

Processing of raw sequences and final dataset construction

Reads were processed using pRESTO [10]. Low quality reads (phred score ≤ 25) were filtered out. IGHV and IGHJ primer sequences were masked and cut off respectively using the *MaskPrimers.py* function, UMI-based consensus sequences created using *BuildConsensus.py* (max.error = 0.1) and paired-end reads assembled. Unique UMI-based consensus sequences represented by at least 3 different UMIs were aligned using IMGT/HighV-QUEST [11]. Functional rearrangements were further processed for germline reconstruction and clonal clustering using Change-O [12].

B-cell receptor repertoire analysis

B-cell receptor (BCR) clonotypes were defined as unique IGHV to IGHJ BCR sequences. Abundance was defined as the number of different UMIs associated with each clonotype, expressed as the percentage of total number of UMIs in the sample. Clonal expansion was calculated as the Gini index on the distribution of the number of unique UMIs per BCR clonotype in each sample, and Clonal diversity as the Shannon index on the distribution of the number of unique BCR clonotypes per clonal lineage in each sample [13]. These indices were calculated using the *renyi* function in the vegan R package (version 2.5-6) [14]. Analysis of somatic hypermutation was performed using the SHazaM R package (version 0.2.1) [12].

ADA testing

Serum was tested for presence of ADA against rituximab performing a chemioluminescence drug-tolerant capture ELISA assay using a Meso Scale Diagnostic platform at the clinical immunology laboratory of GlaxoSmithKline Research and Development, Upper Merion, PA, USA.

Dealing with missing data

Three patients were excluded from the final analysis because the baseline PAXGene sample was not collected. For the remaining 28 patients (baseline characteristics in online supplementary table S1), 5 follow-up samples (out of total 112) were not collected and 14 failed BCR amplification or post-sequencing quality control (online supplementary table S2). For analysis of response prediction all patients were included based on the assumption that sample failure represented complete B cell depletion. These samples will be later referred to as *imputed* data. This assumption is supported by the fact that available samples taken at the earlier or later timepoint indeed did show extensive depletion (see figure 2B). No change in results was observed if these patients were, or were not included (figure 3 and figure 4 and online supplementary figure S3). In case of missing DAS28-score (5% of the data), the timepoints concerned were excluded from the analysis. For the analysis of clonality, somatic hypermutation, depletion and re-population after rituximab, patients were included if they had no (n = 18) or one (n = 5) missing follow-up timepoint (total n = 23).

Statistics

Data are presented as mean and standard deviation (SD) or median and interquartile range (IQR) after D'Agostino and Pearson omnibus test for normality. Differences between groups were evaluated using unpaired t-test and one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons post-test for

normally distributed data or Mann-Whitney test and Kruskal-Wallis test followed by Dunn's multiple comparisons post-test for not-normally distributed data. Contingency tables were evaluated with Fisher's exact test. P-values less than 0.05 were considered statistically significant. Graphpad Prism 7 software (Graph Pad, San Diego, CA, USA) was used to perform the statistical tests.

Results

The impact of rituximab on the B cell receptor repertoire persists for up to one year

It has been shown that 4 weeks after treatment with rituximab the peripheral blood BCR repertoire is characterized by more expanded and mutated BCR clones [3]. To confirm and extend these findings, we prospectively analyzed a cohort of 23 (see Methods section 'Dealing with missing data') rituximab-treated RA patients with one year follow-up after treatment.

At one month after treatment, B cells were undetectable in all patients using classical cytometry. Using AIRR, at one month, clonal expansion was significantly higher ($p < 0.001$; 0.52 ± 0.08 vs. 0.27 ± 0.14, mean ± SD; figure 1A), while clonal diversity was significantly lower compared to baseline ($p < 0.001$; 4.75 ± 1.06 vs. 7.53 ± 0.49, mean ± SD; figure 1B). In addition, BCR clonotypes carried a significantly higher number of somatic hypermutations in the IGHV gene ($p < 0.001$; 16 ± 2 vs. 8 ± 3, mean ± SD; figure 1C). These data confirm that one month after treatment with rituximab the BCR repertoire is dominated by fewer, more expanded and more mutated BCR clones.

Further analysis at the follow-up timepoints revealed a trend towards re-establishment of pre-treatment conditions from month 6 post-treatment onwards for all three analyzed indexes. Compared to month 1, the BCR repertoire at month 12 showed significantly higher clonal diversity (5.93 ± 1.20; $p < 0.001$) and less IGHV gene mutations (12 ± 5; $p < 0.05$). Of note, this effect was even more accentuated when excluding re-treated patients at month 6 (online supplementary figure S2). Taken together these results show that rituximab induces profound changes on the peripheral blood BCR clonality. In some patients these changes persist for up to 12 months after treatment while in others restoration of the pre-treatment conditions starts at month 6 after treatment.

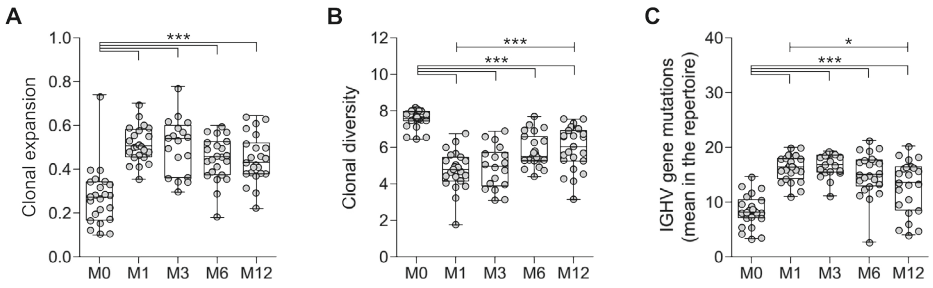

Figure 1 | Peripheral blood BCR repertoire in patients undergoing rituximab treatment

Boxplots showing (A) clonal expansion: Gini index, (B) clonal diversity: Shannon index and (C) the average IGHV gene mutation load in samples obtained before (M0), and at one (M1), three (M3), six (M6) and twelve (M12) months after treatment with rituximab. Boxplots show the median, 25% and 75% interquartile range, error bars show the range, and single data points are depicted in grey (*p ≤ 0.05, ***p ≤ 0.001, using one-way ANOVA).

Defining depletion and repopulation timepoint

The trend to re-establish pre-treatment conditions, observed in the peripheral blood BCR repertoire 6 months post-treatment, might reflect the restoration of the B-cell compartment after B-cell depletion. It has been reported that repopulation of B cells after depletion starts with antigen naïve B cells with reduced somatic hypermutation in the IGHV genes [15,16]. We therefore speculated that the percentage of unmutated BCR clonotypes in the repertoire could be used as a proxy to monitor the fraction of naïve B cells in patients undergoing rituximab therapy.

The percentage of unmutated BCR sequences in the total repertoire significantly decreased one month after treatment compared to baseline ($p \leq 0.001$; 4.33 ± 3.98 vs. 40.0 ± 17.8, mean ± SD, figure 2A). This decrease was at its lowest at month 3, and persisted up to month 12. Of note, when we excluded re-treated patients, the percentage of unmutated BCRs at 12 months did not differ significantly from that at baseline (online supplementary figure S2D). From month 6 onwards we observed an increase in the percentage of unmutated BCR sequences, which at month 12 was significantly different compared to the dip at month 3 ($p \leq 0.01$; 23.1 ± 26.5 vs. 2.54 ± 3.38, mean ± SD). Overall, it was clear that the observed decrease and increase in the percentage of unmutated BCRs in the repertoire was patient specific.

To address this variability, we defined for each patient a *post-depletion* and a *post-repopulation* timepoint as the first timepoint at which the percentage of unmutated BCR in the repertoire respectively drops below and increases again above 4.4%

(mean minus two-times the standard deviation of the unmutated BCR distribution at baseline) (figure 2B, grey and black arrow). Using these definitions, we observed that out of the 23 patients, 14 (61%) reached their *post-depletion* endpoint at month 1, 19 (83%) at month 3, and 23 (100%) at month 6 after treatment (figure 2C, grey line). Regarding B cell repopulation, cumulatively 2 (9%) patients reached their *post-repopulation* endpoint at month 3, 9 (39%) at month 6, and 14 (61%) at month 12 after treatment (figure 2C, black line). In the remaining 9 (39%) patients no *post-repopulation* timepoint could be defined within the follow-up period of 1 year. In conclusion, using this novel approach our data confirm that B-cell depletion is achieved within one month of treatment in the majority of the patients, and show that B cell repopulation takes more than 12 months in one third of the treated patients.

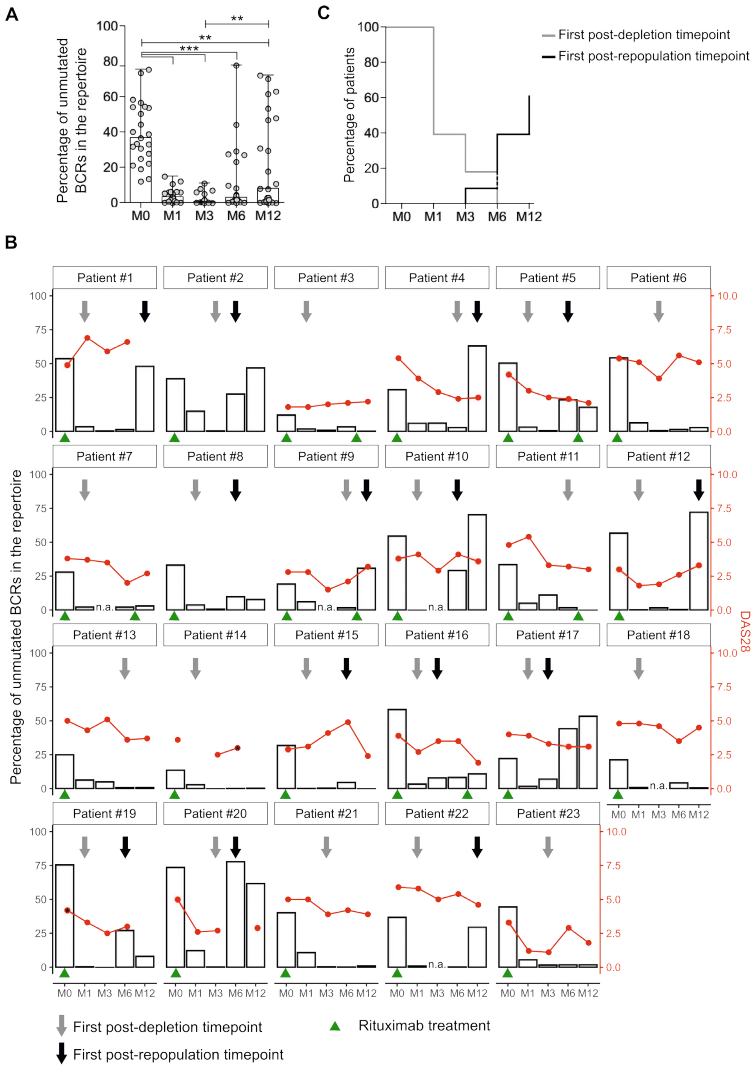

Figure 2 |Monitoring of B cell depletion and repopulation dynamics in peripheral blood BCR repertoire during treatment with rituximab

A) Barplot showing the percentage of unmutated clonotypes in samples obtained before (M0), and at one (M1), three (M3), six (M6) and twelve (M12) months after treatment with rituximab. Bars height shows the median, error bars show the range. Single data points are depicted in grey (**p ≤ 0.01, ***p ≤ 0.001, using one-way ANOVA). B) Disease activity score (DAS28: red dots and line, red scale on the right Y-axis) and percentage of unmutated clonotypes (white bars, scale on the left Y-axis) in the individual patients. Grey arrows indicate the first post-depletion timepoint, while black arrows indicate the first post-repopulation timepoint. Green triangles indicate the timepoint at which each patient received rituximab treatment. C) Percentage of patients that reached the first post-depletion (grey) and first post-repopulation timepoint (black) at the timepoints analyzed.

No correlation of depletion and treatment outcome

To answer the question whether timing of B-cell depletion correlates with treatment efficacy, we evaluated changes in DAS28 and clinical response separately in patients who had their first *post-depletion* timepoint within one month after treatment (early depleting group), and patients who achieved this endpoint later during the follow-up (late depleting group). Interestingly, while achieving the post-depletion timepoint at one month, patients in the early depleting group did not show any change in DAS28 at this timepoint. In contrast, late depleting patients did show a decrease in DAS28-score one-month post-treatment, significantly different from that in early depleting patients (median (IQR): -0.9 (-1.7 – 0.0) vs. 0.0 (-0.7 – 0.2); p-value = 0.04); Figure 3A and online supplementary figure S3A excluding imputed data). This effect was still observed after 3 months (-1.5 ± 0.8 vs. -0.50 ± 1.1; p-value = 0.02; Figure 3B and online supplementary figure S3B). At month 6 or 12 the groups did not show significant differences in changes in DAS28 and in clinical response (data not shown). Taken together these data indicate that timing of depletion is not correlated to long-term therapy response, and suggest that a delayed achievement of B-cell depletion after treatment might be associated with reduced disease activity in the first three months.

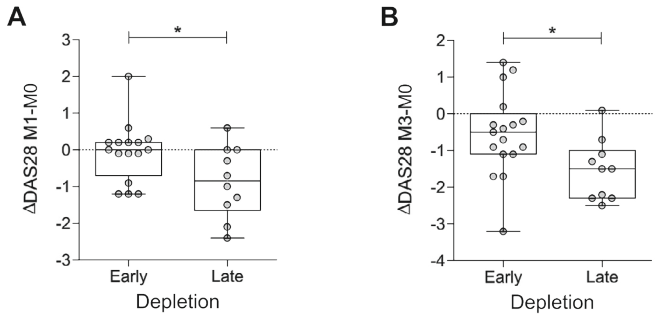

Figure 3 |Timing of depletion does not predict long-term clinical outcome but correlates with short-term disease activity

A-B) Boxplot showing the changes in DAS28-score between month 1 (A) or month 3 (B) post-treatment and baseline in early or late depleting patients Boxplots show the median and 25th and 75th interquartile, error bars show the range, and single data points are depicted in grey (*p ≤ 0.05, using Mann-Whitney test in A and unpaired t-test in B).

Correlation of repopulation with treatment outcome

Next, we evaluated whether the timing of repopulation of the B-cell compartment is correlated with treatment outcome. Patients were divided into an "early" or "late" repopulation group, with early repopulating patients having their first *post-repopulation* timepoint within 6 months after treatment. Compared to baseline, no dif-

ference in DAS28 changes, or in clinical response at month 6 or 12 post-treatment was observed between the two groups (data not shown). However, there was a significant difference when analyzing the change in DAS28-score after repopulation (Figure 4A). In the early repopulating patients, a larger decrease in DAS28-score was measured between month 6 and 12 (p-value < 0.01; early repopulating: -1.17 ± 0.96, late repopulating: 0.18 ± 0.67, mean ± SD). This was also observed when excluding re-treated patients (online supplementary figure S2E) or excluding imputed data (online supplementary figure S3C). Of note, the DAS28 at month 6 did not differ between the two groups (early repopulating: 3.36 ± 0.89, late repopulating: 3.42 ± 1.53, mean ± SD; p-value = 0.92), thus not explaining this observation. In addition, there was no correlation between early depletion and early repopulation (p-value = 0.41), indicating that early depletion does not lead to early repopulation. Thus, early repopulation of the B cell compartment does not predict treatment outcome at 6 or 12 months, but is associated with improvement of disease activity shortly after repopulation.

Effect of anti-drug antibodies on repopulation

ADAs are known to increase drug clearance due to the formation of ADA-drug complexes, and patients who develop ADAs show a reduced drug trough concentration in serum [17,18]. We therefore assessed whether development of ADAs against rituximab was associated with early B cell repopulation. In our cohort of 28 patients, 14 developed ADAs within 12 months of treatment. No association between ADA development and depletion (early or late) was observed (data not shown). Ten (71%) out of these 14 ADA positive patients showed B-cell repopulation within 12 months, while 4 out of 13 (30%) of the ADA negative patients did (p-value = 0.06, n.s., 1-sided Fisher exact test; Figure 4B and online supplementary figure S3D. For one patient no repopulation status was available). Hence, even though not significant, there still might be a trend in favor of an association between the development of anti-drugs antibodies within 12 months of treatment and repopulation of the B cell compartment within the same time-span.

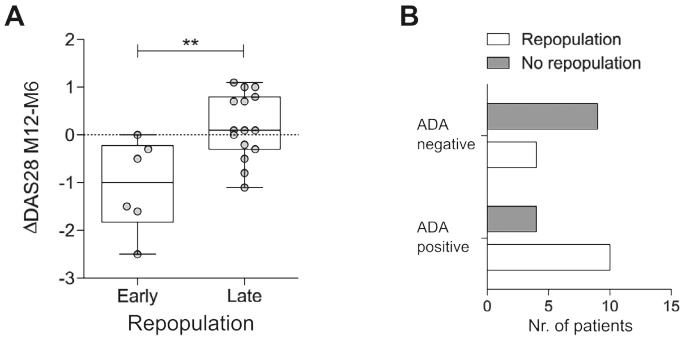

Figure 4 | Repopulation is associated with improvement of disease activity shortly after and possibly with anti-drug antibodies development
A) Boxplot showing the changes in DAS28-score between the month 6 and month 12 after treatment in early or late repopulating patients. Boxplots show the median and 25th and 75th interquartile, error bars show the range, and single data points are depicted in grey (** $p \leq 0.01$, using unpaired t-test). B) Barplot showing the correlation between anti-drugs antibodies (ADA) development and repopulation within 12 months of treatment.

Discussion

Using UMI-based adaptive immune receptor repertoire (AIRR) sequencing in RA patients undergoing rituximab treatment we show that deletion and recurrence of the unmutated BCRs, associated with the naive repertoire, proved a sensitive marker for depletion and repopulation. Using this parameter, we find a correlation between repopulation and clinical improvement shortly after, strongly suggesting that it might be the repopulation, rather than the depletion, that has a dampening role on disease activity.

In this study we monitored unmutated clonotypes in the BCR repertoire with quantitative UMI-based sequencing to sensitively follow the dynamics of B cell depletion and repopulation in rituximab treated patients. Using this method, we observed that *repopulation* of the BCR repertoire within 6 months of treatment did correlate with significantly better disease amelioration in the subsequent period, between month 6 and 12 post treatment, compared to patients that did achieve repopulation later or not at all (p-value < 0.01). This observation was not explained by a difference in disease activity at 6 months between the two groups (p-value = 0.92) nor by a 2nd cycle of treatment. This might indicate that it is in fact the repopulation following rituximab – rather than depletion itself - that is able to “re-set” the (pathological) B cell compartment, leading to temporal improvement of the disease activity. In this context, the ability to promptly recognize the start of B cell repopulation after

depletion might thus be extremely useful in clinical practice, allowing the physicians to more closely monitor disease progression during repopulation and eventually take an informed decision on intensification of treatment.

Using the same approach, we observed that *depletion* does not predict clinical response at 6 and 12 months. On top of that, we showed that patients that achieve depletion within one month of treatment did not show any improvement in disease activity in the same time-span. This confirms previous reports showing that timing and depth of B cell depletion does not correlate with clinical response to rituximab during 0.5 and 1 year of follow-up [4,19]. This lack of correlation is thus not due to a lack of sensitivity of previously used methods. The observed association of early BCR repertoire depletion with *less* response at 1 and 3 months after rituximab therapy is interesting. This might be the result of the fact that in our cohort late depleting patients had a slightly higher baseline DAS28 score (late depleting: 4.6 ± 0.9 vs. early depleting: 4.1 ± 1.1; p-value = 0.2, n.s.), thus resulting in more potential to improve in disease activity [19,20]. However, if indeed an influx of naïve, unmutated B cells explains the clinical effect of rituximab, an alternative explanation might be that part of the patients have a higher baseline influx of naïve, unmutated B-cells before treatment; this increase in B-cell turnover might explain both later depletion with better amelioration of disease activity, and earlier repopulation with better disease amelioration. In case of rituximab, this would lead to a complicated relation between anti-drug antibodies (ADA) development and clinical response. In patients newly starting on rituximab, some will develop ADA after 3 to 6 months [6]. In these patients, ADA is likely to be correlated with earlier clearance of the drug and earlier repopulation (p = 0.06 in our data) leading to better clinical response. However, in patients with pre-existing ADA, these antibodies will interfere with the primary effect of rituximab, i.e. B-cell depletion, and therefore lead to clinical non-response. In this situation the net effect of both mechanisms will determine the clinical response in each individual patient.

This study has several limitations. One is the relatively short follow-up time. Since most of the patients start to repopulate their B cell compartment at 6 months post-treatment, having the last follow-up point set at 12 months post treatment was relatively short to detect disease relapse. The second limitation is that the protocol did allow intensification of treatment in cases of insufficient response. However, clinicians did not have access to the results of the repopulation analysis, and the decision to retreat with rituximab after 6 months did not correlate with early repopulation (p = 0.7). It would have been nice to correlate the BCR repertoire in paired synovial tissue samples with that in peripheral blood to study the recurrence of B

cell clones during disease relapse. Such a study could prove that rituximab does not eradicate pathological B cells but just prevents them to reach the site of disease activity, i.e., the synovium, therefore explaining why - despite the temporary amelioration of disease symptoms - CD20-depleting therapy does not cure RA.

Conclusions

Using AIRR-sequencing to monitor the dynamics of B cell depletion and repopulation after rituximab treatment we observed that timing of depletion and repopulation does not predict response after 6 or 12 months. However, repopulation within 6 months did significantly correlate with response in the subsequent interval (6-12 months), suggesting it is repopulation rather than depletion that influences long-term remission. Based on our results, we hypothesize that interindividual differences in clinical response to rituximab are defined by patient-specific differences in B-cell turnover. We propose further studies are indicated to validate the latter observations, analyze the underlying mechanisms and assess which cell populations are involved.

Supplementary data

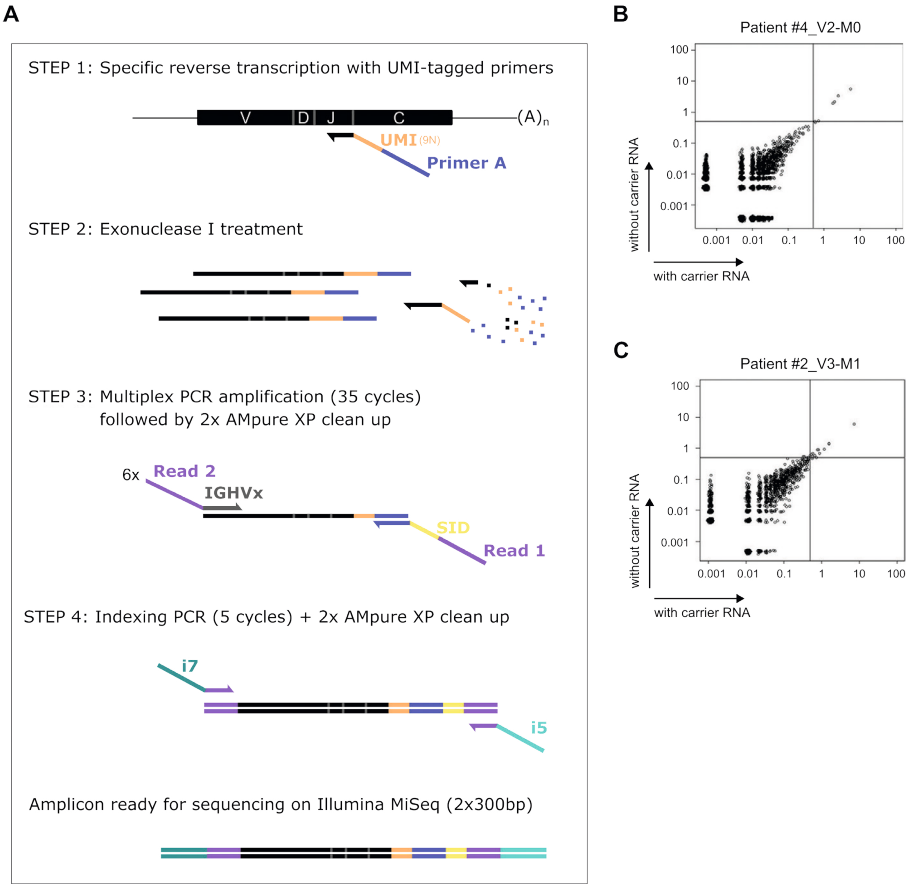

Figure S1 |
A) Schematic representation of the high-throughput UMI-based BCR repertoire sequencing workflow. Specific reverse transcription of BCRh RNA molecules is performed with UMI-tagged primers complementary to the BCR heavy chain joining gene (step 1). Exonuclease I treatment is performed to remove unbound reverse transcription primers (step 2) before performing a multiplexed PCR with 6 BCRh variable (IGHV) chain forward primers and a single reverse primer (step 3). Obtained amplicons are then indexed with i7 and i5 Nextera Illumina indexes (step 4) and sequenced on an Illumina MiSeq platform. B-C) Scatter plots depicting the clonal overlap in the same pre-treatment (B) or post-treatment (C) sample amplified with or without addition of carrier RNA from the non BCR-expressing cell line HEK939T prior cDNA synthesis. X- and Y-axes depict the frequency of each BCR clonotype in the repertoire as percentage of total UMIs.

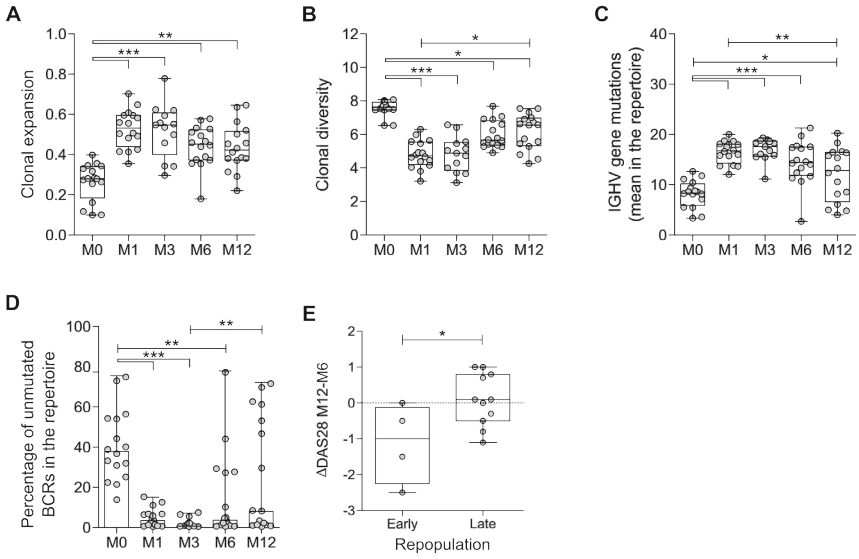

Figure S2 | Results excluding patients who received a 2nd cycle of treatment
A-C) Boxplots showing (A) the clonal expansion: Gini index, (B) the clonal diversity: Shannon index and (C) the average IGHV gene mutation load in samples obtained before (M0), and at one (M1), three (M3), six (M6) and twelve (M12) months after treatment with rituximab. Boxplots show the median, 25% and 75% interquartile range and error bars show the range. Single data points are depicted in grey. D) Barplots showing the percentage of unmutated clonotypes before (M0), and at one (M1), three (M3), six (M6) and twelve (M12) months after treatment with rituximab. Bars height shows the median, error bars show the range. Single data points are depicted in grey. E) Boxplot showing the changes in DAS28-score between month 6 and month 12 after treatment in early or late repopulating patients. Boxplots show the median and 25th and 75th interquartile and error bars show the range. Single data points are depicted in grey. (*p ≤ 0.05, **p ≤ 0.001, using Kruskal-Wallis test in A and B, one-way ANOVA in C and D and unpaired t-test in E).

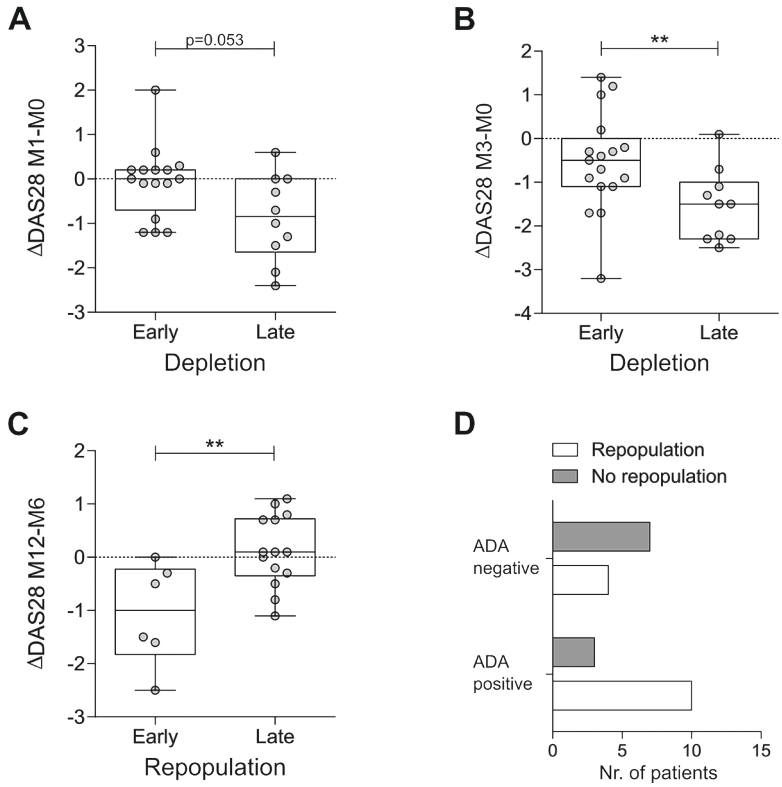

Figure S3 | Results excluding imputed data on depletion and repopulation
A-C) Boxplot showing the changes in DAS28-score between month 1 (A) or month 3 (B) post-treatment and baseline in early or late depleting patients and (C) the changes in DAS28-score between the month 6 and month 12 after treatment in early or late repopulation patients. Boxplots show the median and 25th and 75th interquartile and error bars show the range. Single data points are depicted in grey (** $p \leq 0.01$, using unpaired t-test in B and C). D) Barplot showing the correlation between anti-drugs antibodies (ADA) development and repopulation within 12 months of treatment.

Table S1 | Patients baseline characteristics

Demographics	n=28
Median age, years (range)	61 (21-79)
Female, no. (%)	22 (79)
Never smoker, no (%)	12 (43)
Disease status	
Median disease duration, years (range)	12 (1-28)
IgM-RF positive, no (%)*	20 (74)
ACPA positive, no (%)**	20 (80)
Median DAS28 (IQR)*	4.2 (2.6-6.8)
Median CRP, mg/L (IQR)*	5.2 (1.2-109)

IgM-RF, IgM-rheumatoid factor; ACPA, anti-citrullinated protein antibodies; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein. * one missing data (n=27); ** three missing data (n=25)

Table S2 | List of analyzed samples

	Timepoint				
Patient	Baseline (M0)	Month 1 (M1)	Month 3 (M3)	Month 6 (M6)	Month 12 (M12)
# 1					
# 2					
# 3					
# 4					
# 5					
# 6					
# 7					
# 8					
# 9					
# 10					
# 11					
# 12					
# 13					
# 14					
# 15					
# 16					
# 17					
# 18					
# 19					
# 20					
# 21					
# 22					
# 23					
# 24					
# 25					
# 26					
# 27					
# 28					

Black: not collected; Gray: BCR failed

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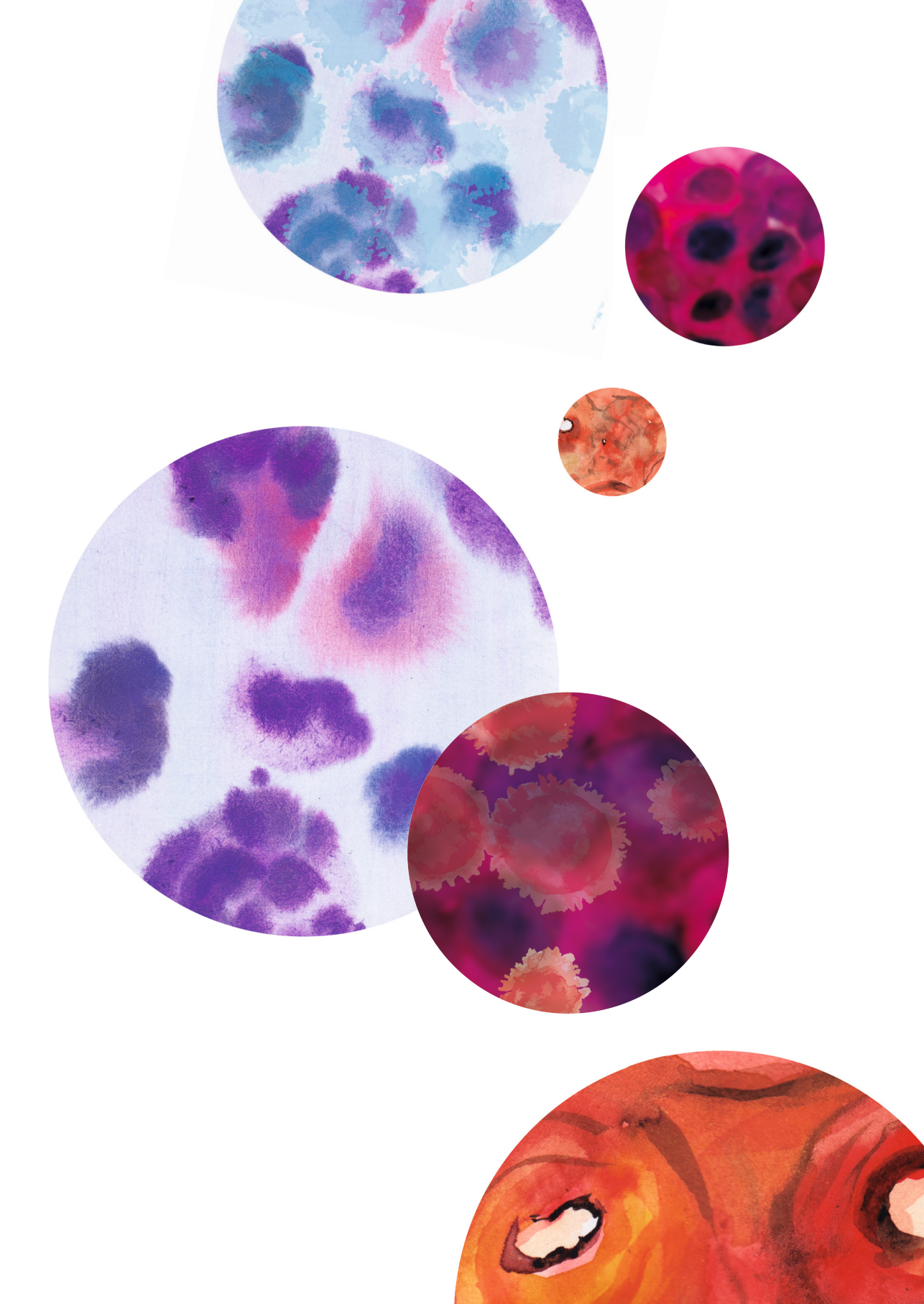

Dynamics of the B cell receptor repertoire during the preclinical phase of rheumatoid arthritis: Longitudinal studies in untreated RA-risk individuals (DOMINO study) and the effects of rituximab treatment (PRAIRI study)

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Manuscript in preparation

6

Abstract

Background B cells play a key role in the development of rheumatoid arthritis (RA), again illustrated by the fact that a single dose of rituximab administered during the RA-risk phase delays onset of arthritis. However, it is not clear whether rituximab (RTX) influences the B cell receptor (BCR) repertoire in this phase, and how this correlates with onset of arthritis.

Objectives To study the effect of rituximab influences on the BCR repertoire during the preclinical phase of RA, and how this correlates with onset of arthritis.

Methods Adaptive immune receptor sequencing (AIRR-seq) of the BCR-heavy (BCRh) chain was performed on peripheral blood samples obtained from RA-risk individuals that were treated with RTX or placebo (PRAIRI study). In a second cohort of un-treated RA-risk individuals (DOMINO study), AIRR-seq was performed on sequential peripheral blood samples and FACS-sorted phenotyped subpopulations.

Results In the preclinical phase of RA Rituximab induces clonal BCR depletion in sequential peripheral blood samples, followed by a gradual re-establishment of the normal BCRh repertoire from 6 months onwards up to 12 months after treatment. Phenotypic analyses in the DOMINO cohort show that plasmablasts/plasma cells carry most of the dominant BCRh signatures. Interestingly, the clonal BCR signatures that initially are dominant at baseline are encoded by memory B cells after 6 and 12 months in peripheral blood.

Conclusion Our results show that the BCRh repertoire constantly changes over time in the preclinical phase of RA. These changes were seen not only after B cell depletion therapy, but also in placebo treated or untreated RA-risk individuals. During the preclinical phase, the most dominant BCRh clones appear to be of the plasmablast or plasma cell phenotype and intriguingly, disappear over time from the plasmablast/plasma cell compartment and reappear as low frequency memory B cell clones.

Introduction

In rheumatoid arthritis (RA), B lineage cells play an important role in disease development. They produce characteristic autoantibodies such as rheumatoid factor (IgM-RF) and anti-citrullinated protein antibodies (ACPAs) which are linked to a more persistent and destructive disease course. Consequently, B lineage cells have been identified as a therapeutic target, and treatments like the anti-CD20 monoclonal antibody rituximab (RTX) that results in B cell depletion have been developed, are effective and are currently widely used in established RA [1,2]. RA may be preceded by a phase in which autoantibodies are detectable in peripheral blood and individuals suffer from arthralgia, with no clinically evident arthritis [3–5]. A small portion of these so-called RA-risk individuals will develop arthritis in the short term [6]. In RA-risk individuals, the PRAIRI study demonstrated that a single dose of rituximab significantly delayed onset of arthritis [7]. It is yet unclear if there is a certain risk signature associated with progression from RA-risk to RA and/or response to rituximab. We recently demonstrated in RA patients that non-response to rituximab was associated with an incomplete disruption of the B cell receptor (BCR) repertoire, with certain BCR clones persisting throughout the therapeutic effect range [8]. These findings prompted us to investigate the effects of rituximab on the BCR repertoire during the at-risk phase. We had the unique opportunity to do so, by evaluating the BCR repertoire behaviour in RA-risk individuals who were treated with RTX or placebo in the randomized controlled PRAIRI study [7]. This phase-IIb randomized controlled trial (RCT) demonstrated that treatment with a single infusion of rituximab in the pre-clinical phase of RA results in a delay in arthritis development of 12 months compared with placebo treatment at the point when 25% of the subjects had developed arthritis [7]. Additionally, we conducted a study in a novel cohort of at-risk individuals not treated with rituximab to determine the phenotype of B cells expressing the most frequent BCR signatures, allowing for detailed insight into B cell clonal changes in the RA-risk phase during the natural course of disease development. Here, we report for the first time the effects of rituximab on the BCR repertoire during the preclinical phase of RA and the dynamics and phenotype of B cells that express the most dominant BCR signatures during this time.

Materials and methods

Patients

From 2010 until 2015 individuals with a positive ACPA and/or IgM-RF status and arthralgia, and at least one of the following features: CRP > 0.6 mg/L or subclinical synovitis assessed by ultrasound or by MRI, but no clinical signs of arthritis were

recruited at the Amsterdam University Medical Centers and Reade, in Amsterdam, the Netherlands into the 'Prevention of Rheumatoid Arthritis (RA) by B cell directed therapy'-trial (PRAIRI-trial, EudraCT Number: 2009-010955-29). In total 81 RA-risk individuals were included. RA-risk individuals in the rituximab group received rituximab 1000 mg i.v. on day 1 (RTX-group). Individuals in the placebo group received an infusion of matching placebo (placebo-group). In addition, all individuals in both groups on day 1 received 100 mg methylprednisolone i.v. as pre-medication to minimize infusion-related reactions of rituximab. During the screening visit, after blood was drawn and at least 4 weeks before baseline, all patients were vaccinated with a pneumococcal vaccine and dependent on the season also with an influenza vaccine. Moreover, some patients got a H1N1 vaccination due to the 2009 flu pandemic. Therefore, the main difference between the screening visit and the baseline visit is that at screening all patients were medication-naïve and not yet vaccinated. In 75 patients (93% of the total cohort), blood was drawn during the screening and baseline visits, after 6 and 12 months and in case arthritis developed, from which RNA was isolated for BCR repertoire analysis. One patient was excluded from BCR analyses due to the fact that she was diagnosed with chronic lymphocytic leukaemia after one month.

Additionally, a study was conducted in a novel cohort that included individuals double positive for ACPA and IgM-RF with arthralgia, but without clinical signs of synovitis in the Amsterdam University Medical Centers from 2016 onwards, the "DOMINant clones in the Onset of RA"-study (DOMINO study). These individuals were not treated with rituximab or placebo and did not receive vaccinations prior to study inclusion (untreated-group). Individuals were followed-up with 6-monthly visits on which fluorescence activated cell sorting (FACS) was performed with subsequent sorted fraction and whole blood BCR sequencing. For this manuscript, all RA-risk individuals that attended at least 2 visits were selected (N=10). 9 individuals that were autoantibody negative, did not have arthralgia and did not have concomitant diseases or drug use were included as healthy controls. The BCR repertoire of these individuals was sequenced, but FACS was not performed. The studies were approved by the independent Medical Ethics Committee of the Amsterdam UMC, location AMC and performed according to the Declaration of Helsinki. All patients gave written informed consent.

Flow cytometry

Plasmablast/Plasma cells were identified as CD3-CD20-CD27+IgD-CD19+CD38++ and memory B cells as CD3-CD20+CD27+IgD-CD19+CD38-. To avoid artifacts, e.g. death of plasmablasts induced by freezing, cells were freshly isolated from peripheral

blood, stained, sorted as plasmablast/plasma cells or memory B cells and directly lysed to capture RNA. In the same patients, these analyses were done at different time points (every 6 months) to study potential phenotypic changes in B cell subsets as well as the stability of dominant B cell signatures over time. These analyses were done based on the sorting and sequencing strategy shown in supplementary figure 2B. The first comparison answers the question whether freshly isolated unsorted PBMCs show a similar clonal pattern as blood collected in PAXgene tubes (and thus immediately lysed). In the second comparison the question is answered to what extent the clonal pattern of plasmablast/plasma cell subset overlaps with the memory B cell subset. The last comparison shows to what extent the PAXgene repertoire is based on the repertoire within plasmablasts/plasma cells and/or the repertoire in memory B cells.

RNA isolation and next-generation sequencing of the BCRh repertoire

RNA isolation from peripheral blood was carried out using PAXgene blood miRNA isolation kit (Qiagen, Hilden, Germany). Complementary DNA (cDNA) of BCR heavy chain molecules was synthesized using a BCR heavy chain (BCRh) Joining reverse primer tagged with a 9 random nucleotide UMI and a consensus sequence, followed by Exonuclease I treatment (Thermo Fisher Scientific, Breda, The Netherlands) to remove left over primers. This was followed by a PCR with forward primers covering the BCRh Variable genes and a reverse primer binding to the consensus sequence previously introduced in the specific-cDNA and tagged with an 8 bp patient identifier (MID, Molecular Identifier). Obtained amplicons were purified using two rounds of AMPure XP beads clean-up (Beckman Coulter, Woerden, The Netherlands), quantified using Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific), dual-indexed with i5 and i7 adapters (Nextera XT Index Kit v2) and sequenced using the Illumina Miseq Kit v3 2 x 300 bp technology according to the manufacturer's manual (Illumina, San Diego, California, USA).

Bioinformatics generation and analysis of BCRh repertoires

The obtained sequencing reads were analyzed with an in-house developed pipeline for repertoire analysis "RESEDA" (REpertoire SEquencing Data Analysis, https://bitbucket.org/barbera/reseda), using the following steps: 1) pairwise assembly of the paired-end reads using PEAR, 2) identification of the 8 bp MID, 3) identification of the complement determining region 3 (CDR3), 4) alignment to the IMGT database to obtain the Variable and Joining gene assignment, 5) removal of reads with low quality bases (Q score < 30) in the CDR3, 6) clustering of reads in clones based on V gene name, J gene name and 100% amino acidic CDR3 identity, 7) UMI-based correction of clonal frequencies and 8) contamination check between samples from different

individuals [9,10]. The final list of clones obtained from the RESEDA pipeline were analyzed with in-house developed R scripts using R version 4.1 using R studio [11,12]. The frequency of each clone was calculated as percentage of the total number of reads with unique UMIs obtained from sequencing of that sample. Clones with a frequency greater than or equal to 0.5% of the total repertoire were labelled dominant or highly expanded clones (HECs) [8]. The impact of a clone was calculated as its UMI-corrected frequency in the repertoire, and the impact of a group of clones as their cumulative frequency.

Determination of B cell repopulation after depletion

After depletion with rituximab, repopulation of B cells in RA patients starts with reappearance in peripheral blood of antigen-naïve B cells with reduced somatic hypermutation in the IGHV genes [13,14]. We decided to use the percentage of unmutated BCRh clones in the repertoire as a proxy to monitor the fraction of naïve B cells in RA-risk individuals undergoing RTX therapy. For each patient, the percentage of unmutated BCRs was determined at all visits. After depletion, repopulation was taken to occur at the time-point that the percentage of unmutated BCRs increased again above the cut-off of 2.9%. This cut-off was calculated as the mean unmutated BCR percentage at screening minus two times the standard deviation.

Statistics

Data and statistical analysis was done with R software (version 3.4.3) and Graphpad Prism software (version 9.0). Differences between groups were analysed using the Kruskall Wallis test after testing for normality. P values <0.05 were considered statistically significant.

Results

Patient characteristics

Patient characteristics at the screening visit from the 74 included RA-risk individuals in the randomized controlled trial (PRAIRI, RTX-group and placebo-group), the 10 RA-risk individuals in the untreated DOMINO group and 9 healthy controls are shown in Table 1. All RA-risk individuals were double-positive for IgM-RF and ACPA. CRP and ESR levels were low and did not differ significantly between the RA-risk groups. From the 74 individuals in either of the intervention groups, 38 were randomized to receive RTX (RTX-group) and 36 were randomized to receive placebo (placebo-group). In total, 29 RA-risk individuals developed RA over time. 27 had received an intervention with RTX or placebo and 2 were untreated. From the 27 treated RA-developers, 13 had been randomized into the RTX-group.

Table 1 | patient characteristics

	RA-risk PRAIRI		RA-risk DOMINO	Healthy controls
	RTX N=38	**Placebo N=36**	**RA-risk – untreated N=10**	**HC N=9**
Age (mean (SD)), years	51.8 (9.3)	50.5 (9.9)	52.9 (12)	39.7 (12.3)
Male (n (%))	13 (34%)	14 (39%)	2 (20%)	4 (44%)
IgM-RF and ACPA both pos. (n (%))	38 (100%)	36 (100%)	10 (100%)	0 (0%)
CRP level at baseline (median (IQR)), mg/L	3.0 (1.3-5.2)	3 (1.0-5.0)	1.5 (0-2.5)	NA
ESR level at baseline (median (IQR)), mm/h	10.0 (5.0-15.3)	10 (5.0-15.8)	7.5 (2-18.3)	NA
Patients who developed arthritis in total (n (%))	13 (34%)	14 (39%)	2 (20%)	0 (0%)
Time to arthritis **(**mean (SD)), months	18.5 (10.8)	12.1 (11.3)	33 (13)	NA

SD; standard deviation, IQR; interquartile range, RF; rheumatoid factor, ACPA; anti-citrullinated protein antibody.

The BCRh repertoire at screening is comparable between the RA-risk groups

To investigate whether the RTX- or placebo- treated and untreated RA-risk individuals are comparable in their peripheral blood BCRh repertoire, an initial analysis of the BCRh repertoire on the RA-risk groups was performed at the time of screening, prior to treatment and associated interventions (e.g. vaccination, methylprednisolone, etc.). Results were compared with repertoires from healthy controls. At the screening visit, 46 individuals in the RTX- or placebo-group had dominant clones (61%). In the untreated group, we detected dominant clones in 6 out of 10 individuals (60%). Seven of the nine healthy controls had dominant clones (78%). The treated RA-risk individuals (PRAIRI: RTX and placebo arm) showed a similar number (1 (0-4.5) vs 1 (0-2), p>0.99) and impact (0.76 (0-4.4) vs 0.72 (0-1.94), p>0.99) of dominant clones compared with the untreated RA-risk group (DOMINO study; Supplementary figure 1A-1C). Within the treated RA-risk individuals, the RTX-group was compared with the placebo-group, but there were no differences in number of dominant clones (1 (0-4.5) vs 1 (0-2), p>0.99) nor impact (0.76 (0-4.4) vs 0.72 (0-1.94), p>0.99). Similar results were found in the placebo-group compared to the untreated group regarding the number of dominant clones (1 (0-2) vs 4.5 (1-9), p=0.07) and their impact (0.72

(0-1.94) vs 4.24 (0.52-10.95), p=0.15 (Supplementary figure 1D-F). When comparing the RA-risk individuals to the healthy controls, a non-significant trend towards a higher number and impact of dominant clones was observed in the RA-risk untreated group (p=0.07) compared to healthy controls (Supplementary figure 1D-1F).

A single dose of rituximab induces a depletion in the BCRh repertoire

Several studies have investigated the effects of rituximab on the B cell repertoire in RA [8,14]. However, it is unknown if and how rituximab influences the B cell repertoire during the pre-clinical phase of the disease. To this end, we investigated the effects of rituximab and compared the effects of the intervention with placebo treated at-risk-individuals.

Six months after rituximab treatment, the number of BCRh clones was significantly lower while the number and impact of dominant BCRh clones was significantly higher when compared to the screening and baseline visits ($p<0.0001$, $p=0.002$, $p=0.008$ respectively) (Figure 1A-1C). Further analysis on follow-up time points suggests a shift towards re-establishment of the pre-treatment BCR repertoire after B cell repopulation: The number and impact of dominant clones at baseline, after twelve months and at onset of arthritis were highly comparable (Figure 1A-1C). To investigate qualitative changes in the BCRh repertoire after rituximab treatment, we used the screening visit as control to determine to what extent the 25 most dominant (top 25) BCRh clones at screening were present in follow-up visits before and after rituximab treatment. Our analysis showed that compared with the screening visit, there was a relatively high top 25 clonal overlap with the baseline visit. However, this overlap was lost in further follow-up visits post-RTX infusion, on 6 months, 12 months and eventual arthritis onset (Figure 1D).

We observed a trend towards an increase in the number of unmutated BCRh clones which became significant 12 months after treatment when compared to the baseline and screening visits, suggesting an onset of B cell repopulation at 12 months after treatment (Figure 1E). However, at 6 months some patients already showed a strong increase in the number of unmutated clones, while others showed almost a complete lack of unmutated clones, pointing to substantial heterogeneity in timing of depletion and repopulation.

Finally, a diversity analysis using Simpson's index revealed that RTX resulted in a marked decrease in the diversity of the BCRh repertoires 6 months after treatment when compared to the baseline and screening visits (Figure 1F). Again, we observed

a trend towards re-establishment to the pre-treatment diversity at 12 months after treatment (Figure 1F).

In summary, RTX causes a quantitative and qualitative change in the BCRh repertoire during the pre-clinical phase of RA. Depletion of the BCR repertoire can be observed at 6 months after single rituximab infusion. From that point onwards re-establishment of the BCR repertoire in peripheral blood occurs, progressing to a normal repertoire 12 months after treatment.

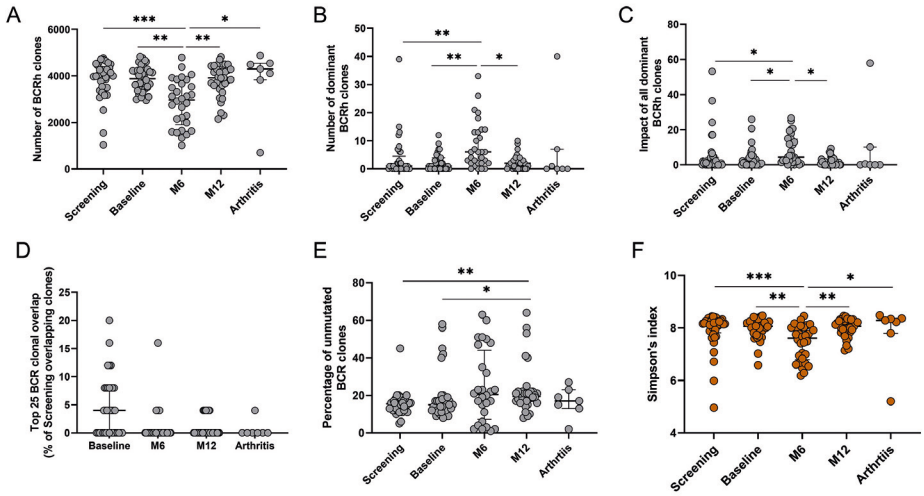


Figure 1 | Influence of rituximab on the BCRh repertoire in RA-risk individuals
Dot plots of (A) the number of BCRh clones, (B) the absolute number of dominant BCRh clones (clonal size ≥ 0.5% of the total repertoire), and (C) impact of the dominant BCRh-clones on the total BCRh repertoire in all RA-risk individuals randomized in the rituximab-arm (n=38). (D) Dot plots of the top 25 clonal overlap between the screening visit and various timepoints after rituximab treatment. (E) Percentage of unmutated BCRh clones in the repertoire at various timepoints before and after treatment. (F) Simpsons index to measure the diversity of the BCR repertoires at various timepoints. dot plots show median and interquartile range and analysed with Kruskall Wallis test. * p value <0.01, ** p value <0.001, *** p value <0.0001

The BCR repertoire is comparable between RA developers and non-developers in the RTX treated group

We next investigated whether the effect of rituximab on the BCRh repertoire during the at-risk phase was different in individuals that eventually developed RA when compared to non-developers. We did not see any significant association in the number of BCRh clones, number and impact of dominant BCR clones, and the progression or non-progression to arthritis (Figure 2A-2C).

Other BCRh repertoire features such as the diversity of the BCRh repertoires, the percentage of unmutated BCRh clones at the timepoint of B cell repopulation after rituximab as well as the persistence of the top 25 most expanded clones at screening did not differ between RA developers and non-developers (Figure 2D-2F).

In conclusion, in the RTX-group the BCRh repertoire is comparable between RA developers and non-developers during the follow-up period.

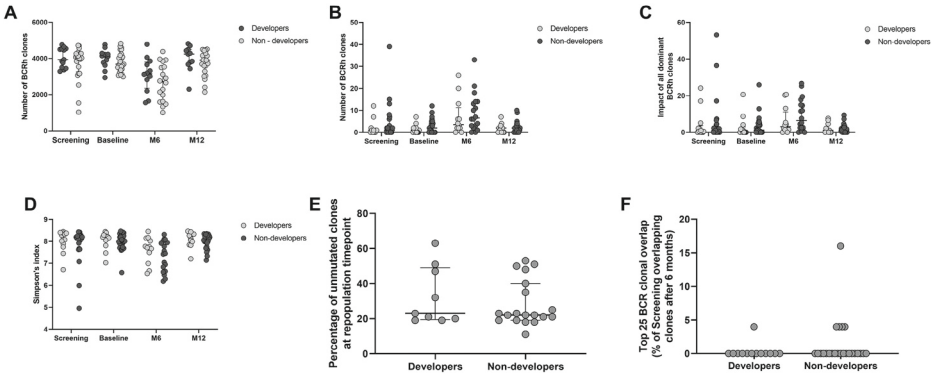


Figure 2 | Influence of rituximab on the BCRh repertoire in RA-risk individuals
Dot plots of (A) the number of BCRh clones during the at-risk phase for individuals that did or did not develop RA, (B) the absolute number of dominant BCRh clones during the at-risk phase for individuals that did or did not develop RA and (C) impact of the dominant BCRh clones on the total BCRh repertoire during the at-risk phase for individuals that did or did not develop RA (D) Simpsons index of the BCRh repertoire during the during the at-risk phase for individuals that did or did not develop RA (E) Percentage of unmutated BCRh clones in the repertoire at various timepoints during the at-risk phase for individuals that did or did not develop RA. (F) Dots plots of top 25 clonal overlaps between the screening visit and 6 months after treatment during the at-risk phase for individuals that did or did not develop RA.

The dynamics of the BCRh repertoire in untreated and placebo-treated at-risk individuals

Since the BCR repertoire of the PRAIRI-placebo-group did not differ from the untreated DOMINO group at screening (Supplementary figures 1D-1F), different time points during follow-up in both groups were compared, to investigate whether the BCRh repertoire changed over time. An additional comparison could be made to estimate the effect of vaccination and methylprednisolone, which were given to the PRAIRI-placebo-group on screening and baseline respectively. Differences in number and impact of dominant clones, throughout follow-up visits, including an eventual arthritis time point did not reach significance (Figure 3A-D and supplementary figure 2A-B). This suggests that the results in the PRAIRI-placebo-treated

group are not likely to be influenced by the vaccination(s) or methylprednisolone they received prior to follow-up visits.

In order to investigate the stability of the BCRh repertoire during the at-risk phase, we tracked dominant clones over time, both in the PRAIRI-placebo-group and in the untreated-group. We calculated the mean number of dominant clones in these groups at the screening visit and determined its standard deviation (i.e. 5). Within each subject, increases or decreases in the number of dominant clones equal or larger than 5 (1 SD) were scored as relevant changes over time. In the PRAIRI-placebo group, 30% of individuals showed a relevant change in the number of dominant clones while 60% of individuals in the untreated group showed a relevant change between the screening and baseline visits (Figure 3E-F). The observed differences in the PRAIRI study occurred largely between screening and baseline visit and may be explained by the vaccinations and/or MPNS treatment given after screening.

Additionally, using the screening visits as control, we analysed to what extent the 25 most dominant (top 25) BCRh clones were also present in follow-up visits. Regarding the placebo-group, a prolonged detectability of certain initial clones could be seen in follow-up visits, which significantly decreased over time (Figure 3G). Up to the arthritis visit, a mean of 3.1% (SD 4.6) of the top 25 clones from the screening visit were still detectable. In the arthritis visit of RA-developers (N=2) of the untreated RA-risk group, a mean of 2.0 % (SD 2.9) of the top 25 clones from the screening visit were still detectable. After 12 months, on average 2.3% (SD 3.9) of the initial top 25 clones were still detectable as top 25 clones (Figure 3H). These results suggest that the majority of the most expanded BCRh clones are replaced by new clones or that the frequency of these clones decreases overtime.

These findings indicate that increases in number of dominant clones can occur without any intervention, suggesting a behavioural pattern in which dominant clones are either not persistently present or may rise/decrease in frequency over time. Dividing the placebo-group into individuals who either did not (n=22; 61%) or did develop arthritis (n=14, 39%), we did not see any significant differences in terms of the stability of dominant BCR clones during the at-risk phase (data not shown).

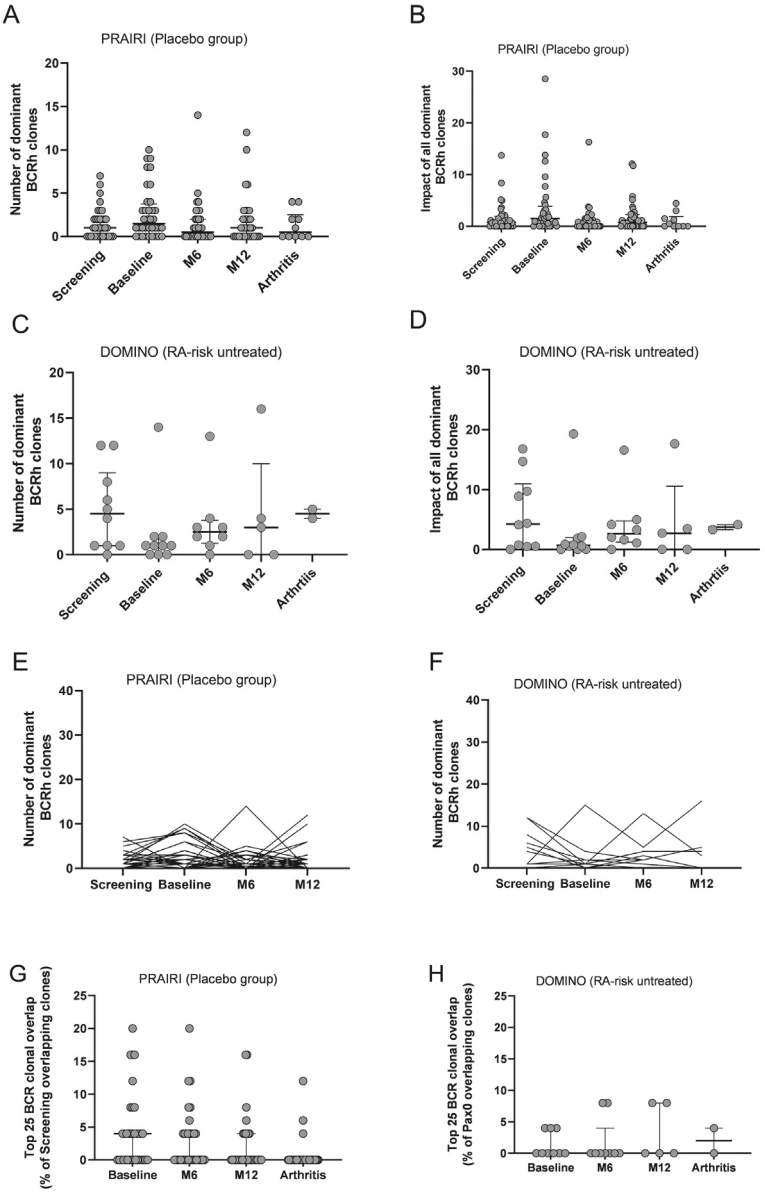


Figure 3| Behaviour of BCRh clones in RA-risk individuals over time

Dot plots of (A-B) the number and impact of dominant BCRh clones in the PRAIRI study. (C-D) the number and impact of dominant BCRh clones in the DOMINO study (E-F) Line plots of the number of dominant BCRh clones over time in the PRAIRI and DOMINO studies. (G-H) percentage of overlapping top 25 BCRh clones in comparison with the screening visit per patient for the PRAIRI study (n=35) and the baseline visit for the DOMINO study (n=10) is shown. Data are presented as median and interquartile range.

Plasmablast/plasma cells dominate the BCRh repertoire during the pre-clinical phase of RA

To determine the phenotype of the (dominant) BCR clones during the at-risk phase, we developed a FACS panel for B cell phenotyping as shown in supplementary figure 3A. As shown in Figure 4A-C, the sequencing results from lysed PBMCs used for the phenotyping of BCR clones are highly comparable to those of lysed peripheral blood cells captured in PAXgene tubes. In 10 RA-risk individuals of the DOMINO study, a total of 23 phenotyping visits were performed. From the peripheral blood collected during these visits, a total of 77 dominant BCRh clones could be detected. FACS sorting allowed us to sequence plasmablast/plasma cell and memory B cell fractions for further downstream sequencing. Of the 77 dominant BCRh clonal signatures in whole blood 88% (n=68) could be retrieved in sequenced data originating from cells with a plasmablast/plasma cell phenotype (Figure 4D-4F and supplementary figure 3C). Fifty percent of these 68 dominant clones could also be retrieved in the memory B cell compartment (Figure 4G-2I). Nine of the 77 dominant BCRh clones detected in peripheral blood could not be found in the plasmablast/plasma cell nor in the memory B cell compartments.

To further investigate the behavior and possible origin of these dominant BCRh clones which were predominantly of the plasmablast/plasma cell phenotype, we looked further into their presence over time. Interestingly, 6 and 12 months after the initial phenotyping visit the dominant BCR clones could no longer be found in the plasmablast/plasma cell compartment, but could be traced back as low frequency clones in the memory B cell compartment (Supplementary figure 4A-3D).

In summary, plasmablasts/plasma cells carry most of the dominant BCRh signatures, while some BCRh signatures are shared across different B cell phenotypes.

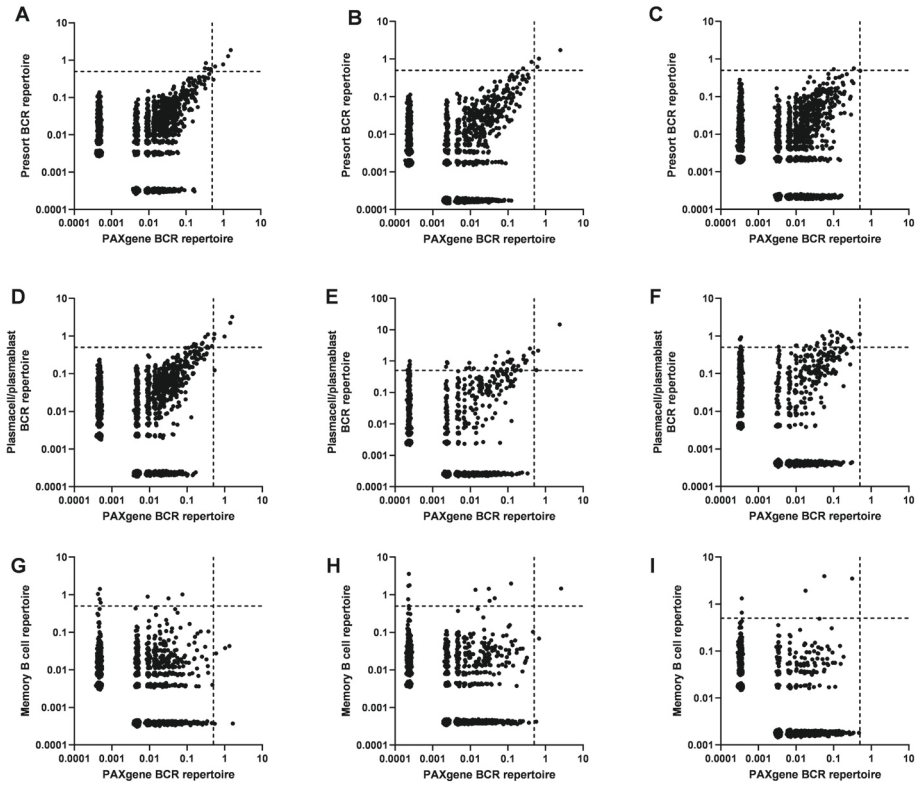


Figure 4 | Phenotyping of BCRh clones in RA-risk individuals
(A-C) CDR3 clonal overlap between peripheral blood (x-axis) and unsorted PBMCS (y-axis) is shown for three patients. (D-F) CDR3 clonal overlap between peripheral blood (x-axis) and sorted plasmablast/plasma cells (y-axis) is shown for three patients. (G-I) CDR3 clonal overlap between peripheral blood (x-axis) and sorted memory B cells (y-axis) is shown for three patients. Each dot represents a unique CDR3 BCR clone, and its frequency in the analyzed repertoire is depicted as percentage of total UMIs. The dotted lines on each axis indicate the 0.5% cut-off for dominant BCR clones.

Discussion

We combined two unique studies performed in RA-risk individuals, yielding novel data in the field of preclinical rheumatoid arthritis. At-risk individuals were randomized and treated with rituximab (RTX) or placebo in the PRAIRI study that demonstrated that RTX delayed the onset of RA [7]. Because the intervention was a single infusion with RTX, in contrast to the majority of clinical trials where RTX is dosed more than once, the course post-intervention can be studied more accurately. The current data demonstrates that the BCRh repertoire of RTX treated RA-risk individ-

uals is fundamentally altered due to effective B cell depletion for up to 6 months after a single infusion of rituximab, which is underlined by a decrease in the number of clones, an increase in number and impact of dominant clones, a decrease in the diversity of the BCRh repertoire and a subsequent increase of unmutated BCR clones, suggesting re-establishment of the repertoire after B cell repopulation. This re-establishment of the repertoire is best visible between 6 and 12 months post RTX-infusion. This is the first time that the effects of RTX are studied in RA-risk individuals. The findings are in line with previous studies conducted in established RA [15].

The RTX-group and placebo-group were very similar in baseline characteristics, but due to the nature of the study, all participants were vaccinated at the screening visit and subsequently received methylprednisolone (MPNS) at the baseline visit prior to the intervention (i.e. RTX or placebo). To evaluate this effect, we established a novel cohort of at-risk individuals with similar inclusion criteria: baseline clinical and clonal characteristics were comparable between all RA-risk individuals although an increased CRP or signs of subclinical synovitis on ultrasound were not necessary for inclusion. When comparing both groups during follow-up, similar dynamics were seen, suggesting that the effect of MPNS or vaccination is limited or worn-off shortly after screening in the PRAIRI-placebo-group.

Unfortunately, no BCRh repertoire related parameters that would potentially associate with RA development could be distinguished in the peripheral blood. In sub-analyses, comparing developers with non-developers in both the PRAIRI-placebo-group and untreated group, the impact and number of dominant clones were comparable throughout follow-up timepoints. In addition, when comparing the RA developers versus RA non-developers in the RTX-treated group, we also did not find any peripheral blood BCRh repertoire parameters that could distinguish both groups. However, it should be noted that this not preclude such differences in other tissues, e.g. in lymphoid tissues such as lymph nodes (LNs) and bone marrow (BM). This could explain the ability of RTX to delay the onset of RA in RA-risk individuals. Such a hypothesis is in line with a previous report that showed changes in various B cell subsets in lymphoid tissues of RA patients treated with RTX [16]. Therefore, future studies aimed at unravelling the effect of RTX treatment on the BCR repertoire in LNs and BM during the RA-risk phase are needed.

A possible explanation for the lack of association of peripheral blood repertoire with onset of arthritis might lie in the observation that the BCRh repertoire is subject to major changes over time. Dominant clones varied from time point to time

point, and the top-25 clones were variable in every follow-up visit. The majority of these dominant clones originate from cells that exhibit a plasmablast/plasma cell phenotype. This is in line with what is known about mature plasmablasts/plasma cells in peripheral blood: they generally have a short survival time in circulation, yet have a high RNA load, which may result in more dominance in the BCRh repertoire [17–19]. On a more general note: It shows that the BCRh repertoire is constantly changing over time, also in the preclinical phase of RA. This is expected, following B cell depleting therapy, but is also present in placebo treated or untreated RA-risk individuals. It suggests a continuous effort to develop new dominant clonal (plasmablast) responses, some of which might be involved in autoantibody production in at-risk patients. Such a continuously renewing response in this phase might explain induction of new (sub)specificities in these autoimmune responses just before the onset of arthritis [20].

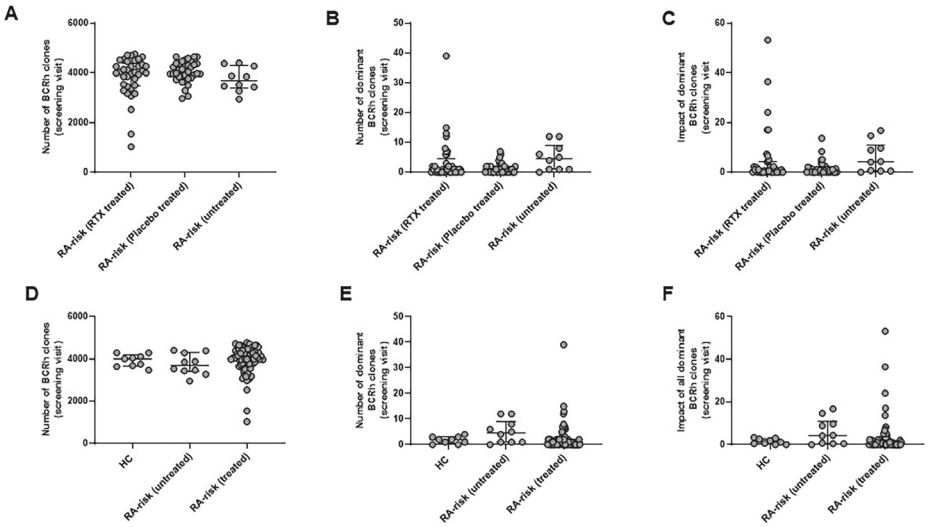
Interesting is how clones that initially were dominant at screening visit or at baseline would switch their phenotype in peripheral blood once they became non-dominant over a 6-month period: they could be preferentially retraced in the memory B cell phenotype compartment. This dynamic is probably the net result of differences in production rates in germinal centres for memory B cells and plasma cells in different phases of the response, differences in survival time of both cell types in blood, and differences in the efflux from blood to lymphoid or peripheral tissues. In the current study, these “switching” clones could potentially be BCRh clones involved in autoantibody production. Bioinformatic techniques coupled with flow cytometric methods and high throughput screenings are needed to isolate and test the specificity of such clones. It is important to also note here that some of the BCRh clones were detected in both the plasmablast/plasma cell subset and memory B cell subset. Both cell types might well derive from common clonal precursors during germinal centre reactions. It seems plausible that memory B cells that have recognized a certain antigen, might thus carry the same BCRh as the plasma blasts/plasma cells that produce antibodies against such an antigen. It seems very unlikely that there is contamination originating from the FACS experiments, although this is a possibility. The two sorted phenotypes differ in three surface markers (CD19, CD20 and CD38), as well as in their subsequent gating strategy. Furthermore, setting up of sorting gates as well as other experiments were performed with great care using state-of-the-art techniques to decrease the risk of contamination.

We were not able to determine the phenotype of nine dominant BCRh clones detected in peripheral blood. A possible explanation could be that these clones are regulatory (Bregs) or of another phenotype and our FACs panel did not permit for

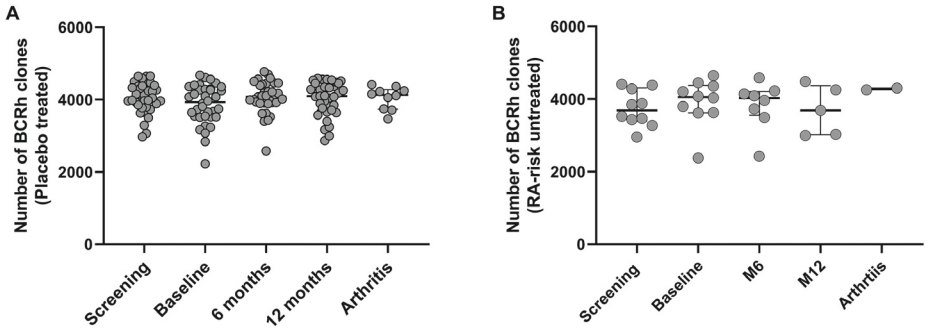
the detection of these cells. Another possibility could be that these clones, although dominant in our RNA analysis, are of low frequency in peripheral blood. Therefore, it could be possible that these cells are found in the blood tubes used for direct lysis and sequencing, but not in the blood tubes used for sorting.

In summary, the current study reveals novel insights into the dynamics of B lineage cells in the pathophysiology and development of RA in the earliest stages of disease development. It shows that the BCRh repertoire is constantly changing over time in the preclinical phase of RA. This is expected, following B cell depleting therapy, but is also present in placebo treated or untreated RA-risk individuals. The most dominant BCRh clones appear to be of the plasmablast or plasma cell phenotype and intriguingly, disappear over time from the plasmablast/plasma cell compartment and reappear as low frequency memory B cell clones in our analyses. This suggests continuous development of new dominant clonal responses, some of which might be involved in autoantibody production in at-risk patients. Such a continuously renewing response in this phase might explain the observation that new (sub)specificities in these autoimmune responses just before the onset of arthritis. More insight in these processes might help to develop novel prevention strategies and targeted therapies.

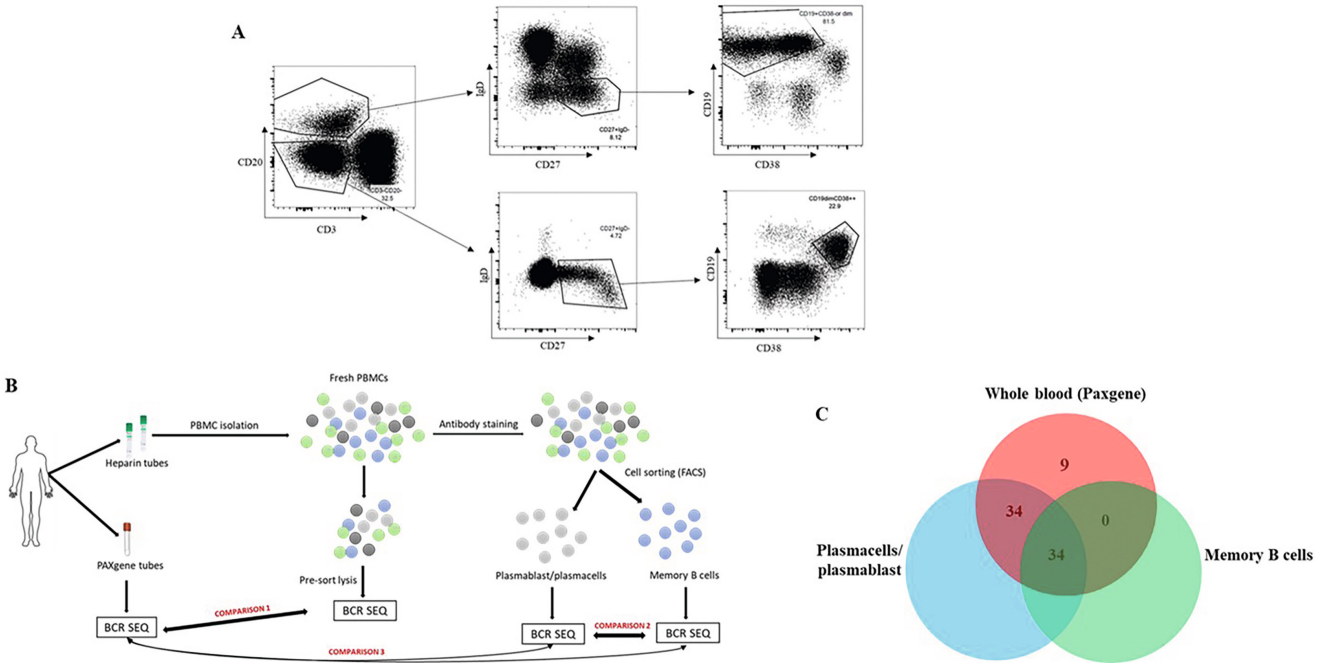
Supplementary data



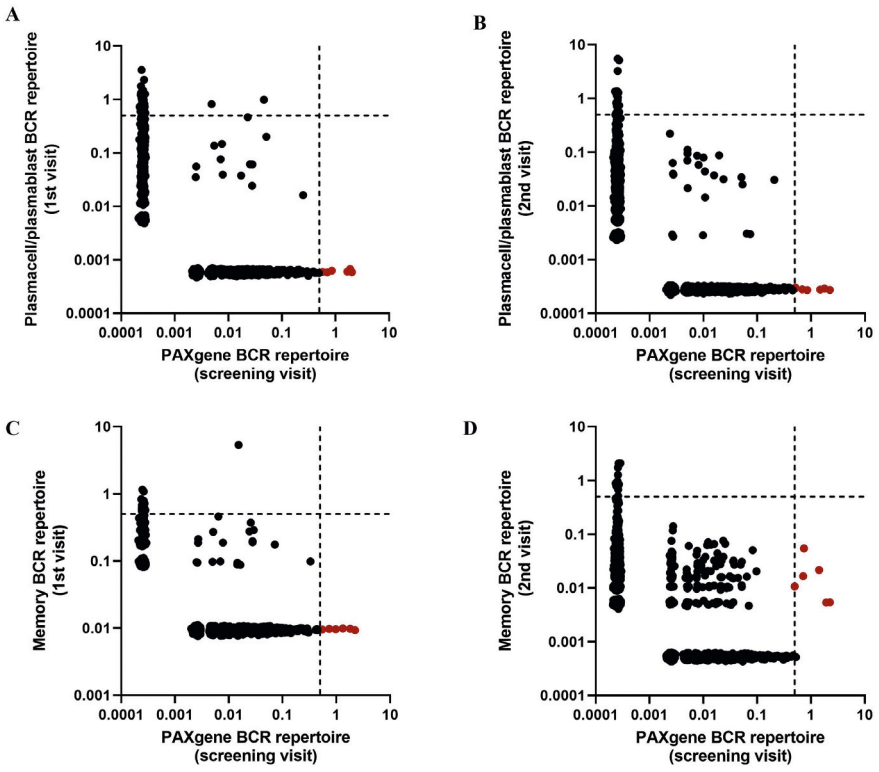
Supplementary Figure 1 |
(A) the number of BCRh clones (B) the number of dominant BCRh clones and (C) the impact of dominant BCRh clones at screening for RA-risk (RTX treated), RA-risk (Placebo treated) and RA-risk (untreated). (D) the number of BCRh clones, (E) number of dominant BCRh clones and (F) impact of dominant BCRh clones at screening in healthy controls and RA-risk individuals. Data are presented as median and interquartile range.



Supplementary figure 2 | The number of BCRh clones during follow-up in (A) the PRAIRI study and (B) DOMINO study. Data are presented as median and interquartile range.



Supplementary figure 3 |
(A) Gating strategy for sorting of plasmablast/plasma cells and memory B cells from PBMCs. (B) Strategy for phenotyping BCR clones in peripheral blood in RA-risk individuals. (C) Distribution of dominant BCR clones found into the various sorted fractions.



Supplementary figure 4 |
(A) CDR3 overlap plot between whole blood BCRh repertoire at screening (x-axis) and the plasmablast/plasma cell repertoire during the 1st phenotyping visit (y-axis). (B) CDR3 overlap plot between whole blood BCRh repertoire at screening (x-axis) and the plasmablast/plasma cell repertoire during the 2nd phenotyping visit (y-axis). (C) CDR3 overlap plot between whole blood BCRh repertoire at screening (x-axis) and the memory B cell repertoire during the 1st phenotyping visit (y-axis). (D) CDR3 overlap plot between whole blood BCRh repertoire at screening (x-axis) and the memory B cell repertoire during the 2nd phenotyping visit (y-axis).

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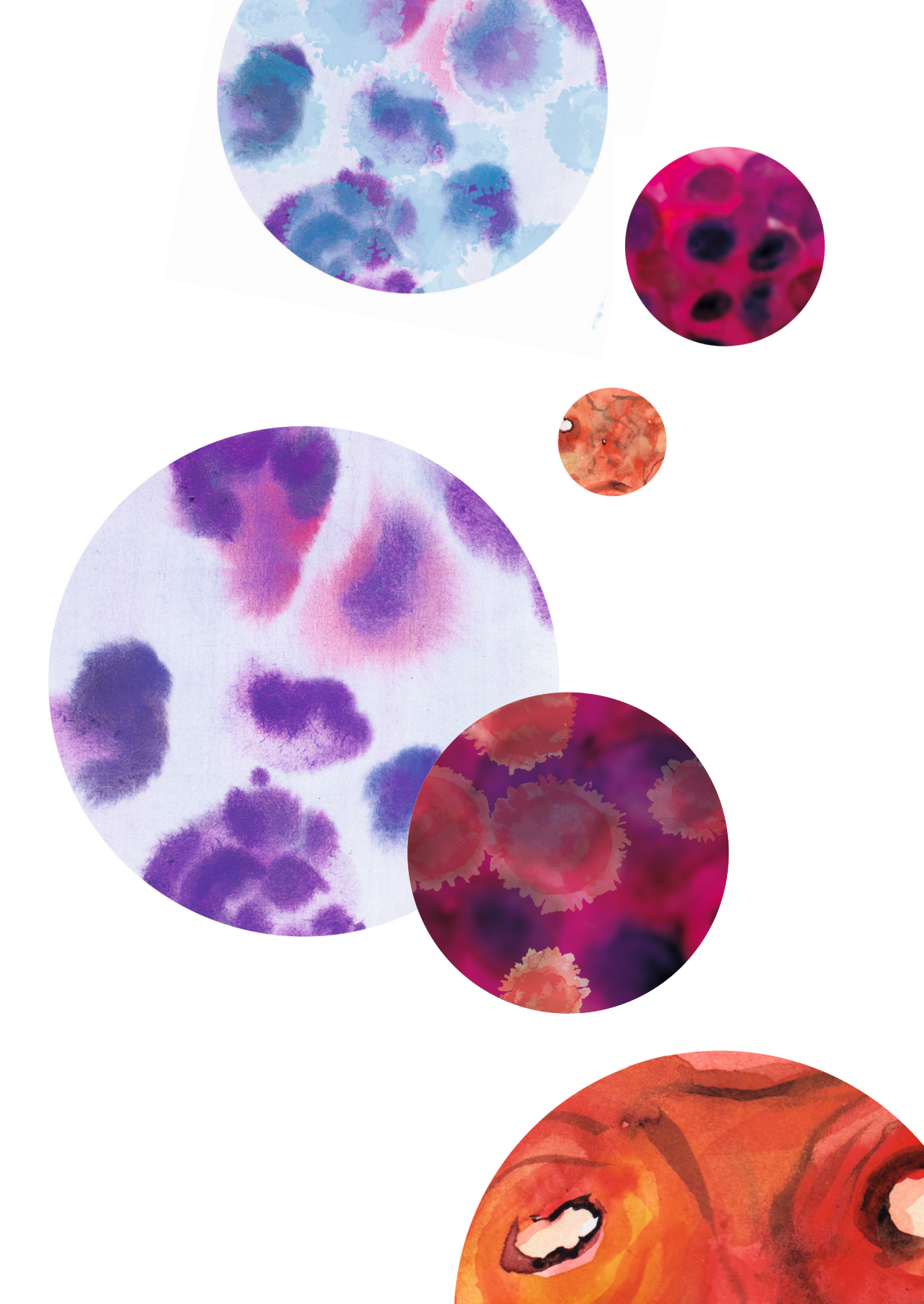

General discussion and future perspectives

7

The therapeutic options for rheumatoid arthritis (RA) have increased immensely in the past two decades and resulted in improvements in outcome and quality of life. Especially, the arrival of biological (b) and targeted synthetic (ts) disease-modifying anti-rheumatic drugs (DMARDs), with therapeutic targets ranging from cytokines (e.g. tumor necrosis factor (TNF) to CD20 on B-cells, has dramatically improved the life of patients as exemplified by improved disease signs and symptoms together with less structural damage. However, these therapies have been proven not to be curative. Therefore, there is a clear need for a better understanding of the underlying pathophysiology, including the contribution of the adaptive immune system and its window of opportunity for treatment in RA patients and individuals at risk of developing RA. Thus, the aim of this thesis was to investigate the adaptive immune response in different phases of RA and in various physical locations. In this final chapter, we will summarize the main findings of each chapter, discuss implications, and share our vision of research challenges in the field for the coming years.

Part I Adaptive immune responses at sites of inflammation

The first part of this thesis describes characteristics of cells involved in the adaptive immune response in RA by investigating T- and B-cell responses in various bodily compartments. In **Chapter 2** we use quantitative, T-cell receptor (TCR) repertoire analysis to characterize RA synovitis in different joints. We demonstrate that RA synovitis is dominated by uniform, systemic T-cell responses. Within a single patient, synovial inflammation in multiple joints was dominated by a limited number of expanded TCR clones, even when these clones were not dominantly present in PB.

In **Chapter 3** we show, using the same quantitative analysis, but now on the B-cell receptor (BCR) repertoire, that dominant B-cell responses are also shared: within the same patient a limited number of expanded B-cell receptor clones were retrieved in the inflamed synovial tissue and fluid in different joints. The observed shared B-cell clones between synovial tissue in different joints indicates that for future B-cell (clonality) studies in RA one might use SF from arthrocentesis as a substitute for ST obtained by arthroscopy or ultrasound- guided biopsy. The latter may have several advantages since arthrocentesis is a less invasive, patient-friendly procedure, which does not require theatre time. However, for functional assays certain differences between SF and ST may exist, which remains to be tested.

Intriguingly, when comparing the B-cell receptor repertoire to the T-cell receptor repertoire, we see some striking differences. In the BCR repertoire the observed ST- SF overlap, mentioned above, is in the range of ST-ST overlap within and between different joints, while the TCR repertoire overlap demonstrated a significantly

higher ST-ST overlap than SF-ST overlap. This might indicate that there is more circulation of B-cells than T-cells from the synovium to the synovial fluid. Since intra-joint overlaps observed in the ST-SF and ST-ST comparisons are in the same range, our data suggest that B-cells derived from synovial fluid or alternatively from synovial tissue are equally informative regarding antigen specificity of B-cells in the synovium. However, more studies are required to further characterize these shared TCR and BCR clones. This might entail functional characterization, including antigen specificity, in order to develop antigen receptor directed therapies. Of note, it might also require additional genomic and proteomic analyses in order to identify more uniform cellular targets selectively present in the activated B- and/or T-cell clones. Isolation of B-cells from ST without modification of the expression of surface markers is challenging, but our studies suggest that the use of SF could be a good alternative. In 2018 Germar et al. performed a proof-of-concept study and were able to immortalize an antigen-specific subset of B-cells (i.e. anti-citrullinated protein antibody-producing B-cells), by genetic reprogramming using B cell lymphoma-6 (Bcl-6) and Bcl-xL proteins, isolated from both blood and SF to study their molecular and phenotypic properties [1,2]. However, this also had its challenges, as only a very limited number of ACPA-specific clones, i.e. three, were stably immortalized, even though many clones (> 300) were generated from 12 RA patients. So, this is a very challenging approach, but not impossible, and it revealed important information on auto-reactive B-cells in RA, more specifically increased CD40 expression and secretion of both pro- and anti-inflammatory cytokines. We believe it to be important to embark on these types of analyses in the near future, preferably in non-transformed cells analyzed directly ex vivo, in order to develop more selective immunotherapy. Aforesaid analyses combined with a selection of the most expanded and shared TCR/BCR clones could shine light on such potential new RA-specific targets.

Furthermore, we noticed that the overlap observed in the top T-cell clones within and between synovial tissue samples taken in the same and in different joints is much more pronounced than the overlap in the B-cell compartment. This is even more clear in the comparison between synovial tissue and peripheral blood. This suggests that B-cell responses might be much more localized than T-cell responses. This might suggest localized proliferation and/or maturation accompanied by an increase in BCR expression, e.g. in the transformation of mature B-cells to plasma cells. Another explanation could be an antigen-driven influx of specific B-cell clones. Such a localized influx of B-cells into the joint on a stable TCR background might result in clinical arthritis. It would be very interesting to further investigate this into more detail, e.g. in a prospective study in seropositive individuals at increased risk of RA which involves sampling of paired ST, SF, and blood in all phases of the

disease. Although, this could be challenging, as sampling ST and SF is difficult when there is no arthritis.

One might also speculate about investigating other bodily compartments where the adaptive immune system thrives, such as bone marrow and lymph nodes. Up till now only one study, from a Swedish group, has been performing single-cell sorting of B-cells from the bone marrow in comparison to peripheral blood in RA patients [3]. They showed sharing of clones between the bone marrow and blood. Furthermore, they showed the presence of heavy-chain so-called "public" bone marrow clones. As four out of five RA patients showed plasma cells in the bone marrow with identical heavy chains but different light chains, this may be indicative of clonal convergence or receptor editing. Studies investigating lymph nodes have been done more extensively [4]. However, it would be interesting to further elaborate on this, by using our quantitative, TCR and BCR repertoire analysis to test for comparability of the various compartments, linking this data to phenotyping, and see over time how the adaptive immune system evolves during disease progression. One might argue that harvesting these samples may be burdensome for patients. However, both ST and lymph node biopsies have been reported to be well tolerated [5–7]. Of note, patients would even be willing to undergo the lymph node procedure a second time, if necessary.

In 2019, Humby et al. showed, using cellular and molecular analyses of synovial tissue in early RA, the presence of three so-called "pathotypes": (1) Lympho-Myeloid, dominated by the presence of B-cells in addition to myeloid cells; (2) Diffuse-Myeloid, with myeloid lineage predominance but poor in B-cells, and (3) Fibroid-Pauci immune, characterized by scanty immune cells and prevalent stromal cells [8]. Elevation of myeloid- and lymphoid-associated gene expression strongly correlated with disease activity, acute phase reactants and DMARD response at 6 months. Furthermore, elevation of synovial lymphoid-associated genes correlated with autoantibody positivity and elevation of osteoclast-targeting genes predicting radiographic joint damage progression at 12 months. A higher proportion of patients classified as Lympho-Myeloid pathotype required biological therapy after 12-months of RA diagnosis [9]. Patients with predominant Fibroid-Pauci immune pathology showed less severe disease activity and radiographic progression [10]. However, this immunopathotype responds significantly less to TNF-blockade [11]. Consequently, a future prospect could be that, before starting anti-rheumatic treatment, a patient would initially undergo synovial tissue biopsy in order to define the pathotype, followed by the best suited treatment (e.g. TNF-blockade, rituximab, tocilizumab) given the most dominant pathotype or gene signature.

Part II Treatment options in early stages of rheumatoid arthritis

In the second part of this thesis, we focus on the earliest phases of RA. In **Chapter 4** we provide a systematic literature review of all preventive strategies explored in at-risk individuals. Several of these preventive strategies have been shown to delay RA onset when applied in the early stages of the disease. Consequently, these therapies decrease disease burden at least temporarily. Unfortunately, to date, true prevention has not (yet) been achieved. In the past years, several clinical trials have investigated the safety and effectiveness of early treatment in RA, focusing on individuals who were at high risk for disease without a clinical diagnosis and patients with very early disease (undifferentiated arthritis (UA) or early RA). The studies described investigate a variety of treatments, such as glucocorticoids, conventional synthetic DMARDs (csDMARDs), bDMARDs, and statins. Some of these therapies are also used in clinical practice for disease management. While glucocorticoids and statins did not have a significant effect on disease prevention, studies focusing on csDMARDs or bDMARDs were able to demonstrate some efficacy of treatment [12–18]. In the TREAT-EARLIER study treatment with methotrexate showed delayed development of RA in individuals with prior high risk of developing RA at 1 year [12]. The PROMPT study, in individuals with UA, showed that this was only in ACPA-positive, but not ACPA-negative [13,14]. Abatacept therapy delayed onset of full-blown RA and showed radiographical improvement and delay of progression, in both at-risk individuals and those with UA [19,20]. The PRAIRI study, in RA-risk individuals, showed that treatment with rituximab resulted in a 12 month delay of RA development [21]. These studies show that early treatment with csDMARDs and bDMARDs could potentially give a transient period of drug-free remission/prevention and reduce physical limitations in both arthralgia, UA, and very early RA patients.

In the meantime, the interim result of the StopRA trial with hydroxychloroquine were published: in at-risk individuals one year of hydroxychloroquine is not superior to placebo in preventing or delaying the development of arthritis and classified RA at 3 years. Thus, the study was halted due to futility [22]. Also, the preliminary results from the APIPPRA study with abatacept were published: during the RA at-risk phase, therapeutic intervention with abatacept for 52 weeks showed a reduction in the development of RA over a two years period [23]. Obviously, further evidence is needed to determine if other disease-modifying therapies can truly prevent or also delay the onset of RA. In the United Kingdom, Emery et al. are currently recruiting those at risk of developing RA to participate in a therapeutic intervention study with baricitinib, a selective Janus Kinase inhibitor, to determine if it reduces the incidence of RA [24].

An important aspect of preventive trials is their feasibility, both in terms of safety, efficacy, and willingness of patients to participate. In this phase, treating individuals has its ethical challenges. At-risk individuals might feel uncertain about the risk of developing disease, as they are still largely asymptomatic, and have to balance an uncertain potential personal benefit in the future against a fear for (potentially) severe side-effects. But also socially, the benefit in terms of cost-effectiveness can be discussed, as some of these treatments are very costly and true prevention has not been achieved yet [25]. On the other hand, a recent extensive survey in three countries showed that at-risk individuals, with an assumed 60% risk of developing RA, are willing to participate in a preventive trial, with treatment efficacy as the most important determinant [26]. Mild side effects were shown to be the least important and the method of treatment administration gave varying results [26,27].

Nevertheless, both the STAPRA study and the PRAIRI trial faced difficulties in patient recruitment, which led to early interruption of the study and a reduction in sample size, respectively [21,28]. This reduction in the number of participants poses a serious problem, because it may lead to loss of statistical significance (type 2 statistical error). Tailored educational strategies, about RA, personal risk, trial aim, and trial medication, might be required to improve individuals' awareness and their predisposition towards preventive studies as patients are more inclined to participate when well-informed and if the risk of RA is strongly reduced by an intervention with limited side effects [25]. Possible options for these educations could be visual overviews showing the study aim, study mechanism, expected benefits, and risks. Furthermore, actively addressing misconceptions and concerns in both treated individuals and aiding physicians. Also, during study design researchers should try and limit study burden as much as possible. The involvement of primary care, through general practitioners (GPs), might help increase inclusion rates. Effectively, GPs see much more patients eligible for inclusion in these trials. Furthermore, GPs could approach individuals at risk of developing RA in the earliest stages, during very early arthralgia or even before UA manifests. Additionally, GPs could identify first degree relatives of RA patients more easily. Patients with early RA on average consult with their GPs four times before being referred to a rheumatologist [29,30]. By then, patients are often not eligible anymore to enroll in a preclinical or early RA study. However, early disease can be challenging to identify in primary care, especially given the fact that RA makes up a small proportion of the non-specific musculoskeletal conditions that account for one in seven GP appointments [31]. For this reason, the establishment of an European registry of at-risk individuals could be of great help to identify and include more patients in RCTs [32].

Of note, a good balance between under- and overtreatment is essential when developing preventive strategies in at-risk individuals, who are strictly speaking not yet patients: There may be substantial adverse effects of disease-modifying therapies, and it should not be assumed that evidence on the balance of benefits and harms found for patients with RA diagnosed following presentation with typical symptoms is generalizable to the at-risk population [30,33]. The time preceding disease manifestation, also known as 'pre-RA', must therefore be recognized as a different entity from RA. Potential harms of a strategy that will label patients as having pre-RA must be considered, such as increased anxiety, reluctance to undertake usual levels of activity due to perceived disability, or wider social implications such as increased costs of insurance policies or restriction of occupational opportunities. The scale of such "potential harms" will depend on the extent of overdiagnosis that can be expected. The optimal primary and secondary care service models to monitor and support patients, and the associated workload and resource implications, also require further research [30].

Potentially modifiable lifestyle risk factors such as increased body mass index and smoking are strongly associated with the development of RA [34]. A recent, other systematic review highlighted that individuals at risk of RA have a need for more knowledge about RA and their potentially modifiable risk factors, which in turn could support their engagement with preventive interventions [35]. However, as yet there is no clear indication that modifying these lifestyle risk factors will prevent or delay the onset of disease [30].

Part III Dynamics in the B-cell repertoire after B-cell depletion in different phases of RA

In the last section of this thesis, we investigated the B-cell repertoire after B-cell depleting therapy in RA patients.

In **Chapter 5**, we demonstrated in RA patients undergoing B-cell depletion using rituximab, that deletion and recurrence of naive B-cells carrying unmutated BCRs proved to be a sensitive marker for depletion and repopulation. We speculated that the percentage of unmutated BCR clonotypes in the repertoire could be used as a proxy to monitor the fraction of naive B-cells. We showed this using BCR repertoire sequencing in RA patients undergoing B-cell depletion therapy with rituximab. Using this parameter, we observed that timing of depletion and repopulation does not predict response after 6 or 12 months. However, repopulation within the first 6 months did significantly correlate with decrease in disease activity in the subsequent period (i.e. 6-12 months after treatment), in comparison with patients who

achieved repopulation later or not at all. This could not be explained by a difference in disease activity at 6 months between the two groups or by the second cycle of rituximab therapy. This might suggest that it is in fact the repopulation following rituximab – rather than depletion itself – that is able to "reset" the (pathological) B-cell compartment, leading to temporal improvement of the disease activity. This observation contrasts with the widely-held view that early repopulation might be a sign of treatment failure, and needs to be confirmed in a larger study. However, it should be noted that earlier studies report opposing results on the correlation between depletion and treatment effect [36]. Further studies are clearly needed in this area.

Unfortunately, this study had a relatively short follow-up time. Since most of the patients start to repopulate their B-cell compartment at 6 months post-treatment, having the last follow-up point set at 12 months post treatment was relatively short to detect disease relapse. During this trial the clinicians were allowed to retreat with rituximab in case of insufficient response. However, the treating clinicians did not yet have access to the results of the repopulation analysis to base their decision for retreatment on, but this may change in the near future.

An earlier study from Humby et al., suggests that patients with low or absent B-cell lineage expression signature in ST respond more effectively to tocilizumab than rituximab [37,38], however this has not yet been validated in an independent cohort. In hindsight, it would have been interesting if we would have also taken ST biopsies from these patients at baseline to be able to stratify patients. Furthermore, paired synovial tissue biopsies and peripheral blood samples would help to study the recurrence of B-cell clones during disease relapse. Such a study could potentially prove that rituximab does not just eradicate (all) pathological B-cells, but rather prevents them to reach the site of disease activity, i.e. the synovium. This might explain why - despite the temporary amelioration of disease symptoms - CD20-depleting therapy does not cure RA.

In **Chapter 6**, we combined two unique studies performed in RA-risk individuals, yielding novel data in the field of preclinical rheumatoid arthritis. In the PRAIRI study at-risk individuals were randomized and treated with rituximab or placebo over time. This study demonstrated that rituximab treatment in the preclinical phase delayed the onset of RA [21]. Since the intervention was a single infusion with rituximab, in contrast to the majority of clinical trials where rituximab is dosed more than once, the course post-intervention can be studied more accurately. The current data demonstrate that the BCR repertoire of rituximab treated RA-risk individuals

is fundamentally altered due to effective B-cell depletion for up to 6 months after a single infusion of rituximab, which is underlined by a decrease in the total number of clones, an increase in number and impact of dominant clones, a decrease in the diversity of the BCR repertoire and a subsequent increase of unmutated BCR clones over time, suggesting re-establishment of the BCR repertoire after B-cell repopulation. This re-establishment of the repertoire is best visible between 6 and 12 months post-rituximab-infusion. These findings are in line with our earlier data in established RA, as described in **Chapter 5**.

In the DOMINO study, a novel cohort of at-risk individuals was established with similar inclusion criteria to the individuals randomized for treatment intervention in the PRAIRI study. This allowed us to investigate the dynamics of the BCR repertoire and dominant clones over time to potentially identify the antigen(s) recognized by the BCRs and involved in the autoimmune repose during the natural course of (potential) arthritis development. This study was also used to determine specificity of BCRs using quantitative BCR repertoire analysis in combination with fluorescence activated cell sorting (FACS) for phenotypical analysis. Unfortunately, no BCR repertoire related parameters that associate with RA development could be identified in the peripheral blood. The BCR repertoire turned out to be very unstable over time, with dominant clones continuously changing from timepoint to timepoint.

Intriguingly, in the at-risk individuals participating in the DOMINO study we saw that once dominant clones, at screening or baseline visit, disappeared over time, they seemed to "switch" to a lower frequency with a memory B-cell phenotype. This could be part of the "secondary immune response" by which memory B-cells recognize an antigen and initiate an immune response. During this secondary immune response memory B-cells are activated via antigen rechallenge and have (at least) two distinct fates: they either differentiate into long-lived plasma cells or enter germinal centers to undergo rounds of population expansion, somatic hypermutation and selection, ending up to restock the memory B-cells pool [39]. In the current study, these "switching" clones could potentially be BCR clones involved in autoantibody production. Bioinformatic techniques coupled with high throughput screening are needed to determine the specificity of such clones. It is important to also note here that some of the BCR clones were detected in both the plasmablast/plasma cell subset and the memory B-cell subset. Whether these clones are in transition from one phenotype to another or have a common precursor remains to be determined. It seems plausible that memory B-cells that have recognized a certain antigen, might carry the same BCR as the plasmablasts/plasma cells that have evolved from these cells and produce antibodies against such an antigen. Furthermore, it would be

very interesting to see whether these "switching" B-cells could also be found or even originate from the synovial tissue. However, the procedure to sample synovial biopsies can be rather difficult in preclinical RA, since patients do not experience arthritis (yet). Therefore, phenotyping of B-lineage cells from the ST in pre-RA will likely be limited.

Concluding remarks and future directions

Over the past twenty years, treatment of rheumatoid arthritis has been dramatically improved. New treatment targets were identified and multiple novel therapeutics became available. This also translated to better control of disease activity and prevention of structural damage in most patients: we hardly see any wheelchairs, nor joint deformities in the outpatient clinic.

However, the three parts of this thesis also reveal new questions worthy of addressing in future research and/or trials. The work done in **Part I** of this thesis shows that in the individual patient shared T- and B-cell responses might be underlying inflammation in different joints. This supports the idea that antigen- and/or receptor-specific therapies targeting a limited set of T-cell and/or B-cell receptor clones might be feasible and effective in patients with rheumatoid arthritis. To further develop this approach of targeted immunotherapy, further characterization of the shared "clones" is indicated (e.g., regarding phenotype, TCR α- and β-chain pairing, Ag specificity, and genomic profiles). Hopefully, novel or improved technologies for single-cell characterization of limited patient samples will rapidly be developed to allow this in the near future.

The results described in **Part II** showed us that early treatment of at-risk individuals may be effective in delaying RA onset, thereby potentially also decreasing disease-related limitations in individuals in the earliest (pre-clinical) phases of RA. Whether this strategy may ultimately lead to prevention of RA remains to be determined. The type of intervention that would be effective may also differ across the different preclinical phases of the disease. As mentioned earlier, investigating various compartments of the immune system could also aid in understanding the immune alterations that are present in the preclinical phase of RA. One can hypothesize that early targeted intervention could result in a larger beneficial effect than the effects of treatment initiated at the time of diagnosis. Numerous components of disease pathogenesis could serve as potential targets for prevention of RA, including autoantibody generation cellular players involved in the autoimmune response, and various other mediators of inflammation. Possible future treatment strategies could be tolerogenic dendritic cell-based therapies through nanoparticles, cellular

therapies with tolerogenic antigen-presenting cells (i.e. dendritic cells), chimeric antigen or auto-antibody receptor (CAR and CAAR, respectively) T-cells, regulatory T-cells and mesenchymal stem cells, immune checkpoint receptor agonists (e.g. PD-1, CD200R or BTLA stimulation) and co-stimulatory molecule blockade, as well as depletion strategies for (subsets of) T and B-cells [40,41].

Based on our results in **Part III**, we hypothesize that interindividual differences in the clinical response to rituximab are defined by patient-specific differences in B-cell turnover in both established and preclinical RA. This could be caused by the different immunopathotypes or gene signatures in the inflamed ST. We propose further studies are indicated to validate these observations, analyze the underlying mechanisms, and assess which cell populations are involved, with a particular emphasis on the B-cells that switch their phenotype over time. This select group of "switching clones" could potentially be involved in autoantibody production and therefore may be of great interest in future studies investigating the B-cell lineage in the preclinical phase of RA, especially in relation to developing new targeted therapies and/or prevention strategies.

Lastly, it is logical to start investigating novel concepts and biological processes such as changes in the BCR/TCR-repertoire over time to elucidate certain pathophysiological aspects in more prevalent diseases, such as rheumatoid arthritis. However, the research performed in this thesis should also be expanded to the "immune-mediated inflammatory diseases (IMIDs)"-group at large, both common (e.g. rheumatoid arthritis, Crohn's disease, spondyloarthropathy, or psoriasis) and rare (e.g. Castleman's disease, hyper-IgD syndrome, dermatomyositis, or relapsing polychondritis). This would give us better understanding of the pathogenesis of these debilitating diseases. Obviously, this is not feasible for one research group and (inter)national collaborations should be sought. Fortunately, many of these partnerships already exist, including the "Autoimmune Research & Collaboration Hub (ARCH)", a Dutch, multi-disciplinary network for systemic auto-immune diseases; the "Rational Use of Biologics in rare Refractory IMIDs Consortium (RUBRIC)" registry, a Dutch online registry for patients with rare IMIDs treated with biologics (www.rubricregistry.nl); and the "Anti-Biopharmaceutical Immunization: prediction and analysis of clinical relevance to minimize the RISK (ABIRISK)" consortium, an international consortium analyzing the mechanisms and consequences of immunization against biologics in several common IMIDs [42–44]. Of note, by including primary health care physicians in (some of) these projects we could gain even more insights, especially in the earlier stages of these diseases.

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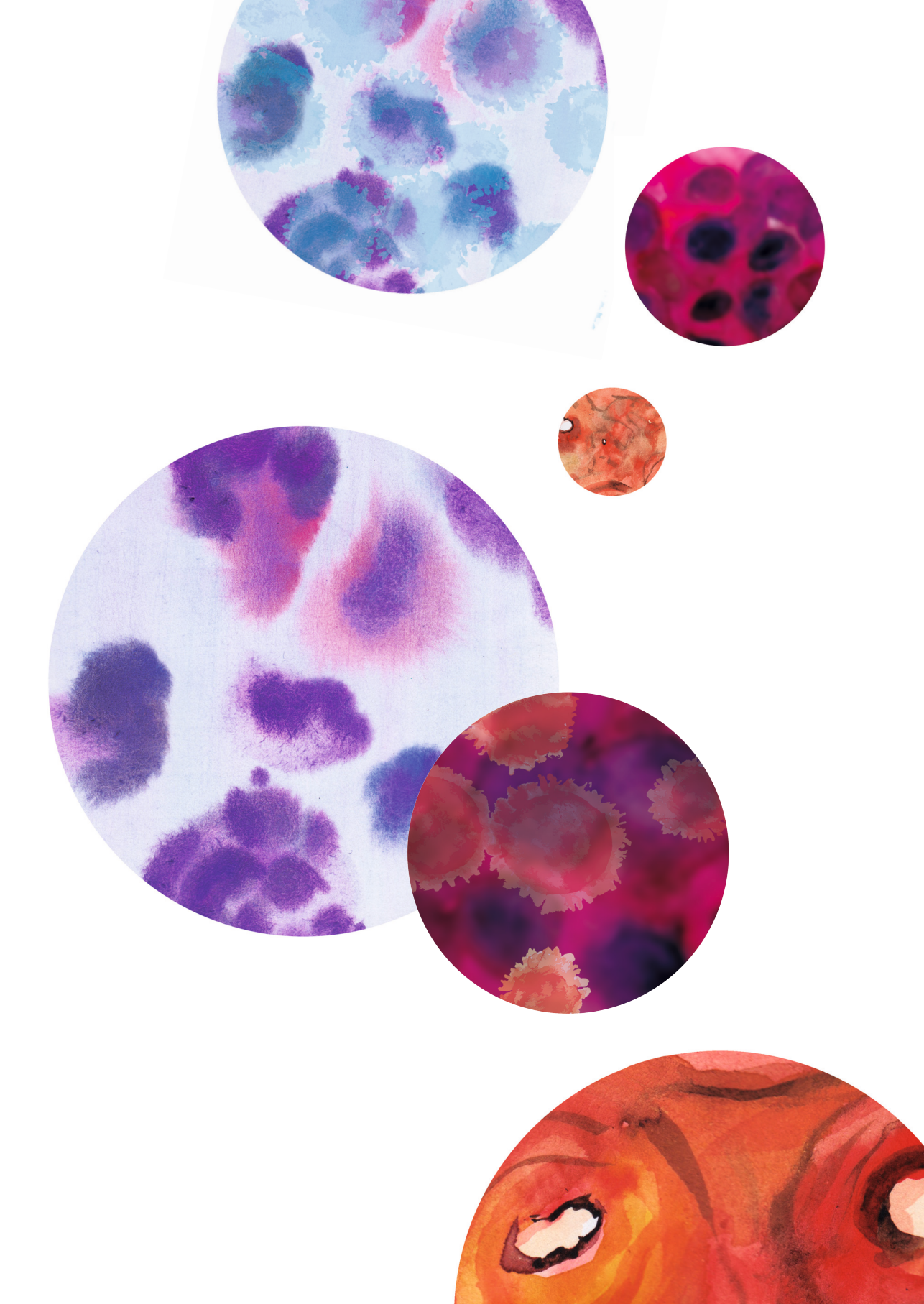
A

Appendices



English summary
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PhD portfolio
List of publications
Contributing authors
About the author
Dankwoord (acknowledgements)

A

English summary

Rheumatoid arthritis (RA) is an autoimmune disease characterized by symmetrical peripheral polyarthritis in the hands and/or feet, leading to long-term disability if not treated effectively. Genetic and immunological studies show that cells of the adaptive immune response are involved in the pathogenesis of RA. The specificity of this immune response is encoded by rearranged T- and B-cell receptors (TCR and BCR, respectively) expressed by clones of T- and B-lymphocytes, plasmablasts, and plasma cells. To investigate the adaptive immune response thoroughly and on a genomic level next-generation sequencing (NGS) technology was developed. This technique makes it able to analyze the repertoire of T- and B-cell receptors individually on the RNA level, in any given bodily compartment, at any given time. Substantial advances in the treatment of RA have been seen, such as the introduction of several new classes of drugs, including biologic Disease-Modifying Antirheumatic Drugs (DMARDs). Targeting the adaptive immune response using these targeted therapies, for instance with abatacept or rituximab, have been proven to be of clinical benefit in RA patients. However, these new therapies are as yet not curative. Relapses may still occur after treatment, and joint destruction is unfortunately inevitable. Selective targeting of such disease-associated adaptive immune cell clones might be highly effective while having few side effects. However, such selective targeting may only be feasible if the same T- or B-cell clones dominate the immune response at different sites of inflammation. Before RA is fully established, it is preceded by a preclinical phase, in which genetically predisposed individuals accumulate environmental risk factors, and during which autoimmunity develops, followed by the emergence of non-specific signs and symptoms before arthritis becomes manifest. It has been shown that early treatment initiation in RA patients improves disease signs and symptoms, with lower disease scores and improved physical functioning as well as reduced structural damage detected by radiography. Early treatment in at-risk individuals has the theoretical potential to delay or prevent disease onset, with a positive impact on both patients' life and society. We investigated the TCR and BCR repertoire using NGS to gain more knowledge on the adaptive immune response in different phases of RA and in various locations; ranging from the early at-risk phase to clinically apparent RA, from studies in blood-only to other bodily compartments, and from T-cells to B-cells.

Part I - Adaptive immune responses at sites of inflammation

Part I describes the behaviour of the adaptive immune responses at various sites of inflammation during RA. We used the earlier-mentioned NGS technology to quantitatively assess whether different T-cell clones dominate the inflammatory infiltrate

at various sites of inflammation in RA, i.e. in blood, synovial tissue, and synovial fluid (**Chapter 2**). In addition, different joints and different locations within one joint are compared. Furthermore, we analyzed to what extent these different compartments share the same dominant T-cell clones. We showed that RA synovitis is dominated by uniform, systemic T-cell responses. Within a single patient, synovial inflammation in multiple joints is dominated by a limited number of expanded TCR clones, even when these clones were not dominantly present in peripheral blood. Since T- and B-cells closely interact in adaptive responses, we consequently started investigating to what extent different joints also share dominant B-cell clones, by investigating the same aforesaid compartments (**Chapter 3**). We demonstrated that dominant B-cell responses are also shared: within the same patient a limited number of expanded B-cell receptor clones were retrieved in the inflamed synovial tissue and fluid in different joints. We conclude that in RA BCR clonal responses may be more localized than TCR clonal responses, pointing to antigen-selective influx, proliferation and/or maturation of B-cells. B lineage cells in the synovial fluid may adequately represent the dominant BCR clones of the synovial tissue, which is in contrast to T-cells. Collectively, the presence of shared B- and especially T-cells in different joints from the same patient suggests that approaches might be feasible that aim to develop antigen-receptor specific targeting of lymphocyte clones in RA as an alternative to more generalized immunosuppressive strategies.

Part II - Treatment options in early stages of rheumatoid arthritis

The second part of this thesis (**Part II**), focuses on the earliest stages of RA (i.e. the at-risk phase, phase of clinically silent autoimmunity, clinically suspect arthralgia, and undifferentiated arthritis), as early intervention in at-risk individuals has the theoretical potential to delay or even prevent disease onset. This initiated, a comprehensive systematic literature review to obtain a complete overview of all preventive strategies applied to at-risk individuals, taking into account all studies that have hitherto been performed and ongoing clinical trials, as well as patient perspectives to understand the feasibility of these types of interventions (**Chapter 4**).

Part III - Dynamics in the of B-cell repertoire after B-cell depletion in different phases of RA

In **Part III** of this thesis, the behaviour of B-cells before and after B-cell depletion is investigated. Although B-cell depleting therapy in RA is clearly effective, response is variable and does not always correlate with B-cell depletion itself. We investigated the depletion and repopulation of B-cells after B-cell depletion with rituximab treatment in RA patients (**Chapter 5**). Time points of achieved depletion and repopulation were defined based on the percentage of unmutated BCR-clones in the repertoire.

Furthermore, the predictive value of early depletion and early repopulation on clinical response was assessed to gain more insight into how this correlates with clinical response. We showed that B-cell deletion using rituximab and recurrence of B-cells carrying unmutated BCRs proved to be a sensitive marker for depletion and repopulation. Using the percentage of unmutated BCR clones, as a proxy for the fraction of naive B-cells, we observed that timing of depletion and repopulation does not predict response after 6 or 12 months. However, repopulation within the first 6 months did significantly correlate with decrease in disease activity in the subsequent period, in comparison with patients who achieved repopulation later or not at all. This might indicate that it is in fact the repopulation following rituximab – rather than depletion itself – that is able to "reset" the (pathological) B-cell compartment, leading to temporal improvement of the disease activity. Importantly, early repopulation of the B-cell compartment did not predict treatment outcome at 6 or 12 months, but was associated with improvement of disease activity shortly after repopulation. Moving forward on this, we focused on B-cells in individuals at-risk of developing RA (**Chapter 6**). We investigated changes in the BCR repertoire over time and the effects of a single dose of rituximab in at-risk individuals. For this, we used unique data from a randomized controlled trial, "Prevention of RA by Rituximab" (PRAIRI) study, and combined this with data from a longitudinal cohort, the "DOMINant clones in the Onset of RA" (DOMINO) study. In addition, a phenotypic analysis of B lineage cells was performed in a similar cohort of at-risk-individuals. The earlier-described PRAIRI study showed that rituximab treatment in the preclinical phase delayed the onset of RA. The current data demonstrated that the BCR repertoire of rituximab treated RA-risk individuals is fundamentally altered due to effective B-cell depletion for up to 6 months after a single infusion of rituximab. This is underlined by a decrease in the total number of clones, an increase in number and impact of dominant clones, a decrease in the diversity of the BCR repertoire and a subsequent increase of unmutated BCR clones over time, suggesting re-establishment of the BCR repertoire after B-cell repopulation. This re-establishment of the repertoire is best visible between 6 and 12 months post-rituximab-infusion. In the DOMINO study, no BCR repertoire related parameters, that would potentially associate with RA development, could be distinguished in the peripheral blood. The BCR repertoire turned out to be very unstable over time, with dominant clones continuously changing from timepoint to timepoint. Furthermore, no differences in dominant BCR clones could be found when comparing individuals who eventually developed RA versus individuals who did not progress towards the disease. However, we did find in the phenotypical analysis that once dominant clones, at screening or baseline visit, disappeared over time, they seemed to "switch" to a lower frequency with a memory B-cell phenotype.

In the final chapter (**Chapter 7**), all findings presented in this thesis are discussed in a broader perspective with relevant clinical implications and possible future directions.

Nederlandse samenvatting

Reumatoïde artritis (RA) is een auto-immuunziekte die wordt gekenmerkt door symmetrische perifere polyartritis in de handen en/of voeten, die leidt tot langdurige invaliditeit als deze niet effectief wordt behandeld. Genetische en immunologische studies tonen aan dat cellen van de adaptieve immuunrespons betrokken zijn bij de pathogenese van RA. De specificiteit van deze immuunrespons wordt gekenmerkt door herschikte T- en B-celreceptoren (respectievelijk TCR en BCR) die tot expressie worden gebracht door klonen van T- en B-lymfocyten, plasmablasten en plasmacellen. Om de adaptieve immuunrespons grondig en op genomisch niveau te onderzoeken, werd "next-generation sequencing" (NGS) technologie ontwikkeld. Deze techniek maakt het mogelijk om het repertoire van T- en B-celreceptoren individueel te analyseren op RNA-niveau, in elk lichaamscompartiment, op elk moment. Er is aanzienlijke vooruitgang geboekt bij de behandeling van RA, zoals de introductie van verschillende nieuwe soorten medicijnen, waaronder biologische Disease-Modifying Antirheumatic Drugs (DMARD's). Het is bewezen dat deze gerichte therapieën, met als focus het adaptieve immuunsysteem, bijvoorbeeld middels abatacept of rituximab, voor RA-patiënten een klinisch voordeel oplevert. Deze nieuwe therapieën zijn echter nog niet curatief. Na de behandeling kunnen terugvallen nog steeds optreden en gewrichtsschade is helaas onvermijdelijk. Selectief doelgericht behandelen van dergelijke ziekte-geassocieerde adaptieve immuuncelklonen zou zeer effectief kunnen zijn, terwijl het weinig bijwerkingen heeft. Een dergelijk selectief, doelgerichte behandeling is echter alleen haalbaar als dezelfde T- of B-celklonen de immuunrespons op verschillende ontstekingsplaatsen domineren. Voordat RA volledig is ontwikkeld, wordt het voorafgegaan door een preklinische fase, waarbij risicofactoren uit de omgeving zich opeenstapelen in genetisch gepredisponeerde individuen, en waarin auto-immuniteit zich ontwikkelt, gevolgd door niet-specifieke kenmerken en symptomen voordat artritis zich manifesteert. Het is aangetoond dat het vroege starten van de behandeling bij RA-patiënten de ziektekenmerken en -symptomen verbetert, met lagere ziektescores en verbetering van lichamelijk functioneren, evenals verminderde structurele schade, zichtbaar op radiologisch onderzoek. Vroegtijdige behandeling van risicopersonen heeft het theoretische potentieel om het begin van de ziekte uit te stellen of te voorkomen, wat een positieve impact heeft op zowel het leven van de patiënt als de samenleving. We onderzochten het TCR- en BCR-repertoire met behulp van NGS om meer kennis te vergaren over de adaptieve immuunrespons in verschillende fasen van RA en op verschillende locaties; variërend van de vroege risicofase tot klinisch manifeste RA, van studies in alleen bloed tot ook andere lichaamscompartimenten, en van T-cellen tot B-cellen.

Deel I - Adaptieve immuunrespons op plaatsen van ontsteking

Deel I beschrijft het gedrag van de adaptieve immuunrespons op verschillende plaatsen van ontsteking tijdens RA. We hebben de eerdergenoemde NGS-technologie gebruikt om kwantitatief vast te stellen of verschillende T-celklonen het inflammatoire infiltraat domineren op verschillende plaatsen van ontsteking bij RA, d.w.z. in bloed, synoviaal weefsel en synoviaal vocht (**Hoofdstuk 2**). Daarnaast werden verschillende gewrichten en verschillende locaties binnen één gewricht vergeleken. Verder hebben we geanalyseerd in hoeverre deze verschillende compartimenten dezelfde dominante T-celklonen delen. We toonden aan dat RA-synovitis wordt gedomineerd door uniforme, systemische T-celreacties. Binnen één enkele patiënt wordt synoviale ontsteking in meerdere gewrichten gedomineerd door een beperkt aantal geëxpandeerde TCR-klonen, zelfs wanneer deze klonen niet dominant aanwezig waren in perifeer bloed. Omdat T- en B-cellen nauw op elkaar inwerken in adaptieve reacties, zijn we daarna verdergegaan met onderzoeken in hoeverre verschillende gewrichten ook dominante B-celklonen delen, door dezelfde bovengenoemde compartimenten te bestuderen (**Hoofdstuk 3**). We toonden aan dat dominante B-celresponsen ook gedeeld worden: binnen dezelfde patiënt werd een beperkt aantal geëxpandeerde B-celreceptorklonen teruggevonden in het ontstoken synoviale weefsel en vocht in verschillende gewrichten. We concluderen dat BCR-klonale responsen bij RA mogelijk meer gelokaliseerd zijn dan TCR-klonale responsen, wat wijst op antigeen-selectieve instroom, B-celproliferatie en/of rijping. B-cellen in het synoviaal vocht kunnen de dominante BCR-klonen van het synoviaal weefsel adequaat vertegenwoordigen, in tegenstelling tot T-cellen. Gezamenlijk suggereert de aanwezigheid van gedeelde B- en vooral T-cellen in verschillende gewrichten van dezelfde patiënt dat, in RA, methodes gericht op het ontwikkelen van antigeen-receptorspecifieke behandeling met als focus lymfocytenklonen mogelijk zijn als alternatief voor meer gegeneraliseerde immunosuppressieve behandelstrategieën.

Deel II - Behandelingsopties in vroege stadia van reumatoïde artritis

Het tweede deel van dit proefschrift (**Deel II**) richt zich op de vroegste stadia van RA (d.w.z. de risicofase, de fase van klinisch stille auto-immuniteit, klinisch verdachte artralgie en ongedifferentieerde artritis), aangezien vroege interventie bij risicopersonen in theorie het potentieel heeft om het begin van de ziekte te vertragen of zelfs te voorkomen. Dit leidde tot een uitgebreide systematische literatuurstudie om een volledig overzicht te krijgen van alle preventieve strategieën die worden toegepast op risicopersonen, rekening houdend met alle onderzoeken die tot nu toe zijn uitgevoerd en lopende klinische onderzoeken, evenals patiëntperspectieven om de haalbaarheid van deze soorten interventies te begrijpen (**Hoofdstuk 4**).

Deel III - Dynamiek in het B-celrepertoire na B-celdepletie in verschillende fasen van RA

In **Deel III** van dit proefschrift wordt het gedrag van B-cellen voor en na B-celdepletie onderzocht. Hoewel B-celdepletietherapie bij RA duidelijk effectief is, is de respons variabel en correleert deze niet altijd met B-celdepletie zelf. We onderzochten de depletie en repopulatie van B-cellen na B-celdepletie met rituximab-behandeling bij RA-patiënten (**Hoofdstuk 5**). Tijdstippen van bereikte uitputting en terugkeer werden gedefinieerd op basis van het percentage niet-gemuteerde BCR-klonen in het repertoire. Verder werd de voorspellende waarde van vroege uitputting en vroege repopulatie op klinische respons beoordeeld om meer inzicht te krijgen in hoe dit correleert met klinische respons. We toonden aan dat het verwijderen van B-cellen met behulp van rituximab en het terugkeren van B-cellen met niet-gemuteerde BCR's een gevoelige marker bleek te zijn voor uitputting en repopulatie. Met behulp van het percentage niet-gemuteerde BCR-klonen, als een proxy voor de fractie naïeve B-cellen, hebben we vastgesteld dat de timing van uitputting en repopulatie geen respons na 6 of 12 maanden voorspelt. Repopulatie binnen de eerste 6 maanden correleerde echter significant met afname van ziekteactiviteit in de daaropvolgende periode, in vergelijking met patiënten die later of helemaal geen repopulatie bereikten. Dit zou erop kunnen wijzen dat het in feite de repopulatie na rituximab is – in plaats van de depletie zelf – die in staat is om het (pathologische) B-celcompartiment te "resetten", wat leidt tot tijdelijke verbetering van de ziekteactiviteit. Belangrijk is dat vroege repopulatie van het B-celcompartiment geen voorspeller was van het behandelresultaat na 6 of 12 maanden, maar geassocieerd was met verbetering van de ziekteactiviteit kort na repopulatie. Voortbouwend hierop, hebben we ons gericht op B-cellen in individuen die het risico lopen om RA te ontwikkelen (**Hoofdstuk 6**). We onderzochten veranderingen in het BCR-repertoire in de loop van de tijd en de effecten van een enkele dosis rituximab bij risicopersonen. Hiervoor gebruikten we unieke gegevens van een gerandomiseerde gecontroleerde studie, de "Prevention of RA by Rituximab" (PRAIRI) studie, en combineerden deze met gegevens van een longitudinaal cohort, de "DOMINant clones in the Onset of RA" (DOMINO) studie. Bovendien werd een fenotypische analyse van de B-cellijn uitgevoerd in een vergelijkbaar cohort van risico-individuen. De eerder beschreven PRAIRI-studie toonde aan dat behandeling met rituximab in de preklinische fase het ontstaan van RA vertraagd. De huidige data toonden aan dat het BCR-repertoire van met rituximab behandelde personen, met risico op RA, fundamenteel is veranderd als gevolg van effectieve B-celdepletie tot 6 maanden na één enkele infusie van rituximab. Dit wordt bevestigd door een afname van het totale aantal klonen, een toename van het aantal en de impact van dominante klonen, een afname van de diversiteit van het BCR-repertoire en een daaropvol-

gende toename van niet-gemuteerde BCR-klonen in de loop van de tijd, wat duidt op herstel van het BCR-repertoire na repopulatie van B-cellen. Dit herstel van het repertoire is het best zichtbaar tussen 6 en 12 maanden na rituximab-infusie. In de DOMINO-studie konden in het perifere bloed geen BCR-repertoire-gerelateerde parameters worden onderscheiden die mogelijk verband houden met de ontwikkeling van RA. Het BCR-repertoire bleek in de loop van de tijd erg onstabiel te zijn, met dominante klonen die voortdurend van tijdpunt tot tijdpunt veranderden. Bovendien konden er geen verschillen in dominante BCR-klonen worden gevonden bij het vergelijken van individuen die uiteindelijk RA ontwikkelden met mensen die geen RA ontwikkelden. We vonden echter in de fenotypische analyse dat zodra dominante klonen, bij screening of baselinebezoek, na verloop van tijd verdwenen, ze leken te "switchen" naar een memory-B-celfenotype met een lagere frequentie.

In het laatste hoofdstuk (**Hoofdstuk 7**) worden alle bevindingen van dit proefschrift besproken in een breder perspectief met relevante klinische implicaties en mogelijke toekomstperspectieven.

PhD Portfolio

PhD student: Anne Musters
PhD period: March 2013 – July 2023
PhD supervisors: Prof. dr. Niek de Vries
PhD co-supervisor: Prof dr. Sander W. Tas

Courses	Year	ECTS
Basic Laboratory Safety *Graduate School AMC, Amsterdam, NL*	2013	0.4
Basic Course Legislation and Organization for Clinical Researchers (BROK/GCP) *Graduate School AMC, Amsterdam, NL*	2013	0.9
Practical Biostatistics *Graduate School AMC, Amsterdam, NL*	2013	1.1
Educational Skills Training *Graduate School AMC, Amsterdam, NL*	2013	0.4
Oral presentation in English *Graduate School AMC, Amsterdam, NL*	2013	0.8
AMC World of Science *Graduate School AMC, Amsterdam, NL*	2013	0.7
Crash course Basic Chemistry, Biochemistry and Molecular Biology *Graduate School AMC, Amsterdam, NL*	2013	0.4
Systematic Reviews *Graduate School AMC, Amsterdam, NL*	2014	0.3
Computing in R *Graduate School AMC, Amsterdam, NL*	2014	0.4
Clinical Epidemiology *Graduate School AMC, Amsterdam, NL*	2014	0.6
Advanced Topics in Clinical Epidemiology *Graduate School AMC, Amsterdam, NL*	2014	1.1
Citation Analysis and Impact Factors *Graduate School AMC, Amsterdam, NL*	2014	0.2
Advanced Topics in Biostatistics *Graduate School AMC, Amsterdam, NL*	2016	2.1
Advanced Immunology *VUmc/Sanquin, Amsterdam, NL*	2014	2.9
Advanced Course in Basic & Clinical Immunology *Scottsdale, USA*	2014	0.7

Seminars, workshops and master classes		
Symposium Cytokine axes in inflammatory bowel disease, psoriasis and spondyloarthritis *AMC, Amsterdam, NL*	2013	0.3
Understanding the value of interventions in rheumatoid arthritis *Prague, Czech Republic*	2014	0.6
Master class by Prof. Dr. Shlomchik *Sanquin, Amsterdam, NL*	2014	0.2

Presentations		
Poster presentation NVR najaardagen	2012, 2015 & 2016	1.5
Stand presentation NVR najaarsdagen	2013, 2014 & 2016	1.5
Oral presentation NVR, session: *Kwaliteit*	2013	0.5
Chairperson EULAR, session: *Basic epidemiology explained*	2014	0.2
Oral presentation ACR, session: *T cell Biology and Targets in Autoimmune Disease*	2015	0.5
Oral presentation EULAR, session: *Novel insights into B and T cell immunity in rheumatic disease*	2016	0.5
Oral presentation NVR, session: *Translationeel*	2016	0.5
Oral presentation EWRR, session: *Crossroads in innate and adaptive immunity*	2017	0.5
Oral presentation EULAR, session: *I've got B in my bonnet*	2018	0.5

(Inter)national conferences		
NVR najaarsdagen *Papendal, NL*	2012-2016	2
ABIRISK annual meeting *Sienna, Italy*	2013	0.5
Investigator meeting Boehringer-Ingelheim *München , Germany*	2013	0.4
Nationaal conferentie zeldzame aandoeningen *Den Haag/Rijswijk, NL*	2013	0.3
Investigator meeting AbbVie *Madrid, Spain*	2014	0.4
Annual meeting European Congress of Rheumatology *Paris, France*	2014	0.9
ABIRISK annual meeting *Brussels, Belgium*	2015	0.5
Annual Meeting American College of Rheumatology *San Francisco, USA*	2015	1.1
Annual meeting European Congress of Rheumatology *London, UK*	2016	0.9
European Workshop for Rheumatology Research *Athens, Greece*	2017	0.9

Teaching and tutoring		
Tutoring bachelor students for thesis *3rd year students Medicine, AMC, Amsterdam, NL*	2013-2015	2
New developments in rheumatology: diagnosis and treatment Course "Klinische en Experimentele Immunologie" *3rd year students Medicine, AMC, Amsterdam, NL*	2016-2017	1

Awards	**Year**
Poster of Merit *Federation of Clinical Immunology Societies*	2014
Travel Grant *Annual European Congress of Rheumatology EULAR*	2016
Travel Grant *Amsterdam Infection & Immunity Institute*	2017
Abstract Award in Basic Science *Annual European Congress of Rheumatology EULAR*	2018

Additional activities		
Out-patient clinic, department of Clinical Immunology and Rheumatology, AMC, Amsterdam, NL	2013-2015	
Mini-arthroscopies, department of Clinical Immunology and Rheumatology, AMC, Amsterdam, NL	2013-2018	
Organizing APROVE science night 2014 & 2015	2013-2015	
APROVE board membership *Chair in 2016-2017*	2014-2017	
AMC Graduate School board membership, Amsterdam, NL	2014-2015	
TOTAL		**31.2**

List of publications

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In rheumatoid arthritis, synovitis at different inflammatory sites is dominated by shared but patient-specific T-cell clones.

Musters A, Klarenbeek PL, Doorenspleet ME, Balzaretti G, Esveldt REE, van Schaik BDC, Jongejan A, Tas SW, van Kampen AHC, Baas F, de Vries N
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Manuscript submitted

Dynamics of the B cell receptor repertoire during the preclinical phase of rheumatoid arthritis: Longitudinal studies in untreated RA-risk individuals (DOMINO study) and the effects of rituximab treatment (PRAIRI study)

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Manuscript in preparation

* Equal contribution

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Off-label voorschrijven van biologicals voor zeldzame immunologische aandoeningen: noodzaak voor goede monitoring via het RUBRIC register
Musters A, Baeten DLP, Tas SW
Nederlands Tijdschrift voor Reumatologie. 2013;4:37-43

Rheumatoid arthritis synovial tissue harbours dominant B-cell and plasma-cell clones associated with autoreactivity
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Anti-interleukin 6 receptor therapy for hyper-IgD syndrome
Musters A, Tak PP, Baeten DL, Tas SW
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About the author

Anne Musters was born in Vlissingen, the Netherlands, on the 8th of April 1987. She grew up living in Middelburg with her loving parents. After graduating from the Nehalennia SG, she moved to Amsterdam in 2005 to attend medical school at the Academic Medical Centre (AMC, University of Amsterdam). Anne combined her studies with several side jobs, e.g. working as a medical secretary and nursing assistant.
From 2008 until 2010 she did a scientific internship at the Department of Neurology under the supervision of Prof. Dr. Y.B.W.E.M. Roos, which strengthened her interest in scientific research. After obtaining her doctoral degree in 2010 Anne went to Cameroon for four months to do a voluntary internship in Tropical Medicine, before starting with the regular internships. In October 2012 she graduated from medical school, cum laude.

Six months later, after traveling through Australia and Asia, she started with a Ph.D. trajectory at the Department of Clinical Immunology and Rheumatology under the supervision of Prof. Dr. N. de Vries and Prof. Dr. S.W. Tas. As a Ph.D. candidate, she was involved in both clinical trials and translational research. She was involved in the setup and implementation of the Dutch rheumatoid arthritis section of an international consortium, namely the "Anti-Biopharmaceutical Immunization: prediction and analysis of clinical relevance to minimize the RISK" (ABIRISK) consortium. Additionally, she worked in the outpatient clinic and performed mini-arthroscopies. Together with Prof. Dr. S.W. Tas, she wrote a treatment guideline for rare diseases for the Nederlandse Vereniging voor Reumatologie (NVR) and a chapter on Castleman's disease for the medical textbook "Rare Rheumatic Diseases", edited by Dr. T.K. Tarrant. Furthermore, she was chair of the Ph.D. association APROVE and a board member of the Graduate School at the AMC.

In September 2018 Anne began her training at the AMC to become a General Practitioner, starting her residency in Haarlem. Currently, Anne is working as a GP in Velserbroek and a penitentiary institution. During the past years, she completed her Ph.D. work on the side.

In 2024, Anne and her family are planning to move to New Zealand to start a new adventure and continue to work as a GP there.

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was dit boekje nooit op tijd af geweest. Tijdens mijn promotietraject zijn jullie allebei geüpgrade tot professor en dat hebben jullie echt verdiend!

Dear paranymphen/fairies, **Giugy and Sabry**, I am incredibly lucky that I have you guys as my colleagues and friends. And I am very happy that you were willing to stand next to me today. It has been a fantastic adventure with a lot of joy, but also some tears and frustrations. But your sharp humor made everything better and easier to endure. I will never forget the trips we made to concerts, festivals, and Italy. Let's continue with that hereafter. Good luck with your theses, one day soon you will also stand here in the Agnietenkapel/Aula. Mille grazie di cuore, bacio!

Lieve **Aramino**, wat heb ik genoten van je blijheid en ik bewonder je doorzettingsvermogen. Met veel plezier kijk ik terug op onze Italië-trip met de Italiaanse meiden. Ik zou zo nog een keer met jullie gaan. En over een paar maanden proosten we op jou!

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