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cDNA phage display for the discovery of theranostic autoantibodies in rheumatoid arthritis

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Conflict of interest

The authors declare that they have no conflict of interest.

Abstract

Rheumatoid arthritis (RA) is the world's most common autoimmune disease mainly characterized by a chronic inflammation of multiple synovial joints. Rheumatologists now have a whole range of treatment options including glucocorticoids (GCs), classical synthetic and biological disease modifying antirheumatic drugs (cs- and bDMARDS), resulting in a tremendous improvement of treatment outcomes for RA patients over the last two decades. Despite this progress, the choice of treatment regimen to achieve stable remission at the individual patient level still largely depends on trial and error. In this review, the need for novel theranostic markers that can predict a patient's response to methotrexate, the standard first-line csDMARD treatment, is discussed. Like in many autoimmune diseases, the majority of RA patients form a whole range of autoantibodies. We aim to find novel theranostic autoantibody markers using Serological antigen selection (SAS), a high-throughput technique that uses cDNA phage display to identify novel antigen targets. We have constructed a barcoded cDNA phage display library from the synovial tissue of three RA patients by fusing cDNA products to the filamentous phage minor coat protein VI. This library contains a large proportion of full length genes and gene fragments that are cloned in frame with the phage gene VI. By screening this library for antibody reactivity in serum samples of patients from the CareRA trial, which compared different intensive treatment strategies based on csDMARDs and a step-down GC schedule, our cDNA phage display library has great potential for the discovery of novel theranostic autoantibody biomarkers.

Introduction

Rheumatoid arthritis (RA) is an autoimmune disorder that mainly affects the peripheral synovial joints. In current clinical practice, the application of an intensive treatment regimen early in the disease process aims at reducing inflammation as soon as possible to avoid damage and functional decline. Despite the fact that treatment options for RA patients have improved enormously the last decades, leading to disease remission in many patients, a true cure is still lacking. Furthermore, for each treatment option, a significant proportion of patients remains non-responsive and finding the right treatment for an individual RA patient still largely depends on trial and error. Therefore, the application of truly personalized medicine in RA is dependent on biomarkers that can predict a patient's response to different therapeutic regimens.

About two thirds of RA patients form autoantibodies, rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA), which are used as serum biomarkers in the diagnostic process and are part of the classification criteria issued by the American College of Rheumatology (ACR) and the European League Against Rheumatism in 2010 [1]. The measurement of circulating autoantibodies is also an important tool in the classification criteria of many other autoimmune diseases such as antiphospholipid syndrome (APS) [2], systemic sclerosis (SSc) [3] and systemic lupus erythematosus (SLE) [4]. The characteristics of autoantibodies explain their popularity as biomarkers: they are easily accessible as secreted molecules in the blood where they can accumulate to high titers, they are stable biomolecules with a high-affinity for their target antigens and show a high disease specificity. With the discovery of ever more autoantibodies with diagnostic value, autoantibody profiles can be determined, allowing for sub-classification of autoimmune diseases, which are often heterogeneous in nature [5-7]. Furthermore, the use of prognostic and theranostic autoantibodies, that respectively predict how disease will progress in particular patients or

whether a patient will respond to a certain treatment, or even predict the most efficient therapy before the intervention is initiated, is gaining increasing attention (reviewed in [8]).

Autoantibodies have been described against most biomolecules including DNA, lipids, carbohydrates and proteins. The subcellular localization of the antigenic targets is not restricted to the cell surface or the extracellular environment, since many autoantigens have been found in the nucleus and the cytoplasm [9]. Autoantibodies against intracellular targets could result from exposure of intracellular antigens upon apoptosis. On the other hand, antibodies generated in response to pathogens can also show cross-reactivity to intracellular antigens via molecular mimicry [10,11]. The presence of autoantibodies extends beyond their role as biomarker since several autoantibodies have been described to be drivers of disease pathogenesis (reviewed in [12]). In the collagen antibody-induced arthritis (CAIA) animal model for RA, antibodies against collagen type II are injected in naïve mice, inducing a rapid-onset arthritis that involves neutrophil invasion, pannus formation and erosions of cartilage and bone in the inflamed paws [13,14]. Although intracellular antibody reactivity has been described, its pathogenic role is not clear. Uptake and transfer to the cytoplasm of antibody-coated virus particles has been shown to induce an innate immune response via the cytoplasmic Fc receptor, tripartite motif-containing protein 21 (TRIM21) [15,16]. However, it is not clear whether such cytoplasmic antibodies could also target intracellular targets in autoimmune disease. Alternatively, autoantibodies formed against intracellular antigens might also function through cross-reactivity with extracellular antigens.

Multiple high throughput methods exist to discover the immunogenic profile against which autoantibodies are formed. Linear epitopes can be determined using technologies such as peptide arrays [17], peptide phage display [18] and phage immunoprecipitation sequencing

(PhIP-Seq) [19,20] while conformational epitopes of novel autoantigens can be found by probing against full length proteins on protein arrays [21] or using cDNA phage display [22]. We have constructed a cDNA phage display library that represents the antigenic repertoire of three different RA patients. This library can be used to identify novel targets of autoantibodies that predict disease progression or therapy response in RA patients. Such prognostic and theranostic biomarkers will allow for a personalized disease management and better treatment outcomes.

RA: a heterogeneous disease based on serological markers

RA is an immune mediated, chronic systemic inflammatory condition mainly affecting the joints [23]. Clinically this disease is characterized by a polyarthritis involving the small joints of hands and feet, but in many cases also the larger joints and sometimes the cervical spine. RA related joint inflammation is primarily situated in the synovium, a thin highly organized structure between the joint capsule and the joint cavity [24], responsible for providing structural support, delivering nutrients to the cartilage and the production of synovial fluid. Morphologically, the inflamed RA synovium is characterized by a mononuclear inflammatory infiltrate that is often well organized and hypertrophy of the synovial lining layer containing fibroblasts and macrophages. If left untreated joint inflammation in RA can lead to destruction of cartilage and bone. Apart from being painful, RA related arthritis leads to considerable loss of joint function, at first directly related to inflammation but later also as a consequence of tissue damage in case of insufficient treatment. The disease burden of patients with RA is however not limited to joint complaints. Many patients with RA suffer from fatigue and they are prone to comorbidities like depression or anxiety, cardiovascular disease and osteoporosis [25-27].

Based on the phenotypical description above, which most patients have in common, one might consider RA to be a disease with a similar, predictable natural evolution leading to a similar treatment response in different individuals, but this is far from true. Already at the clinical level, the syndrome called RA is not so uniform. Apart from signs of inflammation, the serum of a majority of RA patients contains the characteristic markers of autoimmune activation, RF and ACPA. Still, a subset of patients can be diagnosed and even classified with RA based on clinical signs and symptoms without the presence of RF or ACPA. This so-called seronegative RA is more and more considered to be a separate disease entity, sharing phenotypical characteristics with the more classical seropositive RA, but having a completely different underlying pathophysiology with different genetic and environmental risk factors and by consequence also a different natural disease course and responsiveness to treatment [28]. The cellular and molecular background of the differences between seropositive and seronegative RA is actively being explored but until now remains largely unknown.

Traditionally RF/ACPA seropositive RA patients are expected to suffer a more severe disease course in terms of cumulative disease activity, joint destruction, disability, comorbidities and mortality [29]. Moreover, a subgroup of seropositive patients develops extra-articular manifestations of RA such as nodules, interstitial lung disease or vasculitis [23]. RF is an antibody directed against the Fc portion of immunoglobulin G (IgG) [30] and is found in 69-80% of RA patients [31,32] but is also detected in elderly and in other chronic inflammatory conditions limiting RA-specificity to no more than 85% [32]. Binding of RF to Ig-Fc is mediated at an unconventional part of the B cell receptor leaving space for other antigens to be bound and presented to T cells [33]. Immune complexes formed by RF activate B cells and complement, thereby triggering the release of chemotactic factors and recruiting other inflammatory cells [34]. By this pro-inflammatory mechanism of action, RF-positive patients

have a worse outcome [31,35]. Most but not all patients positive for RF, are also positive for ACPA. In contrast to RF, ACPA are highly specific (>95%) for RA with a sensitivity of approximately 70% [32,36]. ACPA are antibodies recognizing citrullinated proteins, which are formed by the post-translational modification of arginine residues into citrulline. ACPA are detected years before symptom onset and have proven to be useful in the prediction of progression from undifferentiated arthritis (UA) to RA [37-39]. Testing for ACPA provides important prognostic information, as ACPA-positive patients are more likely to develop extra-articular manifestations [40,41] and have more radiological progression [42,43]. These observations from clinical assessment were supported by the finding that antibodies against citrullinated vimentin directly bind to the osteoclast surface and induce osteoclastogenesis and bone resorption *in vitro* and *in vivo* [44]. The bad prognostic outcome for ACPA-positive patients requires aggressive intervention as soon as possible, and clinical studies have demonstrated that ACPA-positive and ACPA–negative patients respond differently to treatment with methotrexate [45] or rituximab [46].

Recently, a novel antibody system has been discovered, anti-carbamylated protein (anti-CarP) antibodies, directed towards another post-translational modification [47,48]. Carbamylation is the conversion from lysine to homocitrulline by cyanate. The production of cyanate is induced by myeloperoxidase, released by neutrophils in inflammatory conditions. Higher cyanate concentrations have been detected after smoking, the most prominent environmental risk factor for the development of RA [49]. Although structurally homocitrulline resembles citrulline, and some overlap was found for the two antibody systems, ACPA and anti-CarP recognize their own specific peptides. Anti-CarP antibodies are found in 45% of RA patients and 16-30% of ACPA-negative RA patients, and they are associated with more severe joint damage, also within these ACPA-negative RA patients [47].

Novel autoantibodies in RA were previously identified by serological antigen selection (SAS) [50] and recently validated in two large independent study populations [51]. Antibodies to two Hasselt University (UH) peptides, UH-RA.1 and UH-RA.21, were most promising as they were found in up to one-third of early RA patients. Addition of these novel antibodies to the current diagnostic markers, RF and ACPA, reduced the seronegative population from 38% to 29-32%, and could thus contribute to a better early diagnosis of RA. The potential of combined antibody testing was further illustrated by involving anti-CarP antibodies in the Early Arthritis Clinic (EAC) cohort: anti-CarP status was positive for 12% of early seronegative RA patients, thus reducing the serological gap from 38% to 34%. However, anti-CarP combined with autoantibodies to UH-RA.1 and UH-RA.21 further reduced the gap down to 27%. Next to a diagnostic potential, also correlations with prognostic characteristics were demonstrated: whereas antibodies against UH-RA.1 were related to sustained DMARD-free remission in the EAC cohort, pointing towards a better outcome, antibodies against UH-RA.21 were associated with worse disease based on associations with the presence of erosions, elevated ESR and a higher tender joint count.

Other antigenic targets in RA that have been described, include antibodies directed against arginine deiminase 4 (PAD4), calpastatin, phospholipids, α-enolase, stress proteins (binding immunoglobulin protein (BiP or p68)), glucose-6-phosphate isomerase, heterogeneous nuclear ribonucleoprotein A2/B1 (anti-RA33), v-Raf murine sarcoma viral oncogene homologue (BRAF) and several cartilage proteins (collagen, fibronectin, cartilage oligomeric matrix protein), summarized in [52-55]. Most of these autoantibodies still require validation studies in larger cohorts to determine their exact role.

Different treatment regimens for RA

Treatment of RA patients has become much more successful over the last decades thanks to the development of new treatment strategies and more targeted therapeutic agents called biologicals [56]. The efficacy of RA treatment can be evaluated based on different bio-psycho-social outcome parameters, but biological disease control remains the quintessence of any pharmacological intervention and it is also a prerequisite for psychosocial wellbeing of most individuals with RA. The current biological target of RA treatment is remission, which can be defined in many different ways but is in essence the absence of any significant disease activity [57]. Most rheumatologists agree there is a window of opportunity for successful introduction of RA treatment, probably consisting of the first 3-4 months after symptom onset, during which therapeutic interventions are particularly effective, resulting in better chances of achieving long-term sustained remission, avoiding irreversible biological disease consequences and preserving optimal functionality as well as structural joint integrity [58]. Based on this knowledge, current guidelines prescribe that RA has to be treated early, intensively and to target [59-61].

Methotrexate (MTX) at sufficiently high dosages is considered the initial treatment of choice, but given its slow onset of action it is combined preferably with medication allowing a more rapid control of inflammation. In daily practice, many patients with early RA receive MTX with low dosages of glucocorticoids (GC) for a couple of weeks and therapy is adapted depending on the effect (step-up). On the other hand, so-called Cobra strategies (based on the Dutch acronym “combinatietherapie bij reumatoide artritis”) combining classical synthetic (cs) disease-modifying antirheumatic drugs (DMARD) with a step-down bridge schedule starting from moderate or high dosages of GC were demonstrated to be superior in several randomized controlled trials and perfectly feasible in an observational cohort [62-66]. Still, csDMARDs do

not fully suppress disease progression in all patients and combination therapies are regularly accompanied by an increase in toxicity and adverse effects [67-69].

Inadequate response and toxic effects are the major reasons to switch from csDMARDs to biological (b) DMARDs or biologicals, recombinant protein drugs copying the effects of natural *in vivo* substances. The first bDMARDs were chimeric monoclonal antibodies aimed at tumor necrosis factor α (TNF-α), the major pro-inflammatory cytokine involved in RA pathology and for which targeting in both mice and humans has been shown to ameliorate disease [70-73]. TNF-α inhibitors approved for treatment of RA are infliximab, etanercept, adalimumab, golimumab and certoluzimab, all proven to be highly effective, especially in combination with MTX [74]. New bDMARDs beyond TNF-α inhibitors target cytokine pathways (anakinra, anti-IL-1RA and tocilizumab, anti-IL-6 receptor), B cells (rituximab, anti-CD20) and T cell activation (abatacept, CTLA4-antibody fusion protein targeting CD80/CD86 on antigen-presenting cells) [75-78].

bDMARDs combined with MTX were also shown to be more efficacious than csDMARD monotherapy in early RA, but until now their effectiveness was only rarely directly compared with step-down Cobra strategies [79]. The available evidence suggests that the remission rate and preservation of structural joint integrity on X-ray in patients treated with TNF-α blockers is not superior compared to individuals with early RA treated with high-dose GCs combined with MTX [80,63]. There is however an important price difference between both strategies and until now it remains unclear if bDMARDs can be discontinued without loss of effect after being used temporarily as remission-inducing agents [81]. For these reasons, current guidelines do not recommend bDMARDs as first-line treatment for early RA except in special cases with an unfavorable prognostic profile. Consequently, in many countries

bDMARDs are not reimbursed as first-line treatment for RA and in Belgium they can only be introduced in case of failure of two csDMARDs. Cobra-like treatment regimens therefore seem the obvious first choice for remission induction in early RA but there are still important implementation problems because of practical issues and doubts from patients and doctors related to high GC dosages and the need to combine csDMARDs in these schedules [82-84]. Moreover, it remains unclear if patients with a less severe disease profile according to the present standards, for instance seronegative patients without bone erosions and with low disease activity, should receive the same treatment as patients with a severe risk profile [29,85].

Lack of treatment response for patient subpopulation

Despite the success of current therapeutic strategies, resulting in remission rates of up to 70%, there is still an unmet need in a large proportion of early RA patients. A non-responsive treatment population can be found for csDMARDS treatment regimens [86], and despite their high effectiveness in many patients, bDMARDS such as TNF-α inhibitors also give variable therapy responses and still a considerable number of patients do not respond [87,88]. Of course the remaining important delay in diagnosis and appropriate treatment initiation [89-91], difficulties with the implementation of sufficiently intensive treatment schedules [82-84] as well as adverse reactions to DMARDs, comorbidities, discrepancies in treatment goals between patients and health professionals [89] and psychosocial factors can be held partly responsible for this, but the leading cause of the unmet need remains insufficient biological disease control with current therapeutics. It might be that current therapies are simply not sufficiently potent to deal with the particular pathophysiological processes responsible for persistent disease activity in certain individuals or that they fail to hit the right targets. For this reason, current treatment paradigms still need further improvement and the place of novel therapeutics early in the disease

course needs to be evaluated. An important condition for a cost-effective implementation of such new treatment approaches would of course be a more successful patient stratification.

One of the main reasons why RA treatment is still suboptimal is that the therapeutic response is unpredictable [92]. In practice, many rheumatologists use the same initial treatment strategy for all RA patients, consisting of methotrexate temporarily combined with a low dose of GCs, stepping up to combination therapy or biologicals in case of an insufficient response [65,93] . Even if they attempt to avoid under- or overtreatment in individual patients by taking into account traditional risk factors, they are not very successful at this, probably because the performance of these prognostic markers is reduced in case of an intensive treatment approach such as advocated in the guidelines [94]. This means that finding the right treatment schedule for an individual RA patient is still pretty much a question of trial and error and given the slow onset of effect inherent to many anti-rheumatic therapies, it can take quite some time before sufficient disease control is achieved. This is not only a question of treatment efficacy but also of drug toxicity and side effects at the individual patient level. Inevitably, until today many patients experience prolonged periods of insufficient symptom control, important discomfort and impaired quality of life as a consequence of this empirical treatment approach. Moreover, the delay in initiating the appropriate therapy caused by this trial and error strategy can be a reason for individual RA patients to miss the window of opportunity for effective therapeutic intervention [90,95,96] . This means their chances of achieving sustained disease remission, a prerequisite for optimal functionality and structural joint integrity, are compromised and they are more at risk for comorbidity, chronic disease behaviour and other psychosocial consequences of the disease.

A more personalized treatment approach based on a more effective patient stratification would not only be of benefit to individual patients and their doctors, but would probably also be more cost-effective from a societal point of view, even considering the potential extra costs for the introduction of expensive novel therapies earlier in the disease course in the minority of RA patients at risk of an insufficient response to intensive treatment regimens based on csDMARDs and GCs like the Cobra Slim regimen [56]. Currently, the traditional risk factors, even when used in matrices, are not very helpful in this respect [92]. The classical diagnostic markers, RF and ACPA have been studied extensively for their theranostic potential in the context of MTX treatment. For RF status, no predictive value of MTX therapy response has been found and despite some conflicting results, also ACPA status before the onset of treatment does not seem to correlate with the effectiveness of MTX (reviewed in [97]). Some studies have reported a lower treatment response for ACPA-positive patients but these might reflect the higher disease activity of ACPA-positive patients. Recently, a number of studies have reported on novel biomarkers to predict the treatment outcome of MTX therapy. Quantification of T-cell subsets using fluorescence-activated cell sorting (FACS) of 108 early RA patient samples at baseline showed that remission in response to MTX monotherapy was associated with a higher frequency of naïve CD4+ T-cells compared to healthy controls [98]. Recently, Peres et al described [99] that MTX treatment response can be predicted at baseline using flow cytometric determination of the ectonucleotidase CD39 on the cell surface of regulatory T-cells (Tregs). Patients that did not reach remission after MTX monotherapy (disease activity score DAS28> 4,0) express a significantly lower level of CD39 on Tregs compared to healthy controls and MTX-responsive RA patients. These studies are able to distinguish between MTX responders and non-responder using flow cytometry and contribute to the biological understanding of treatment response. However, a serological marker would be more easily applicable in clinical routine testing. The acute phage protein haptoglobin is locally elevated in RA synovial fluid

and arthritic tissue. Moreover, baseline haptoglobin serum levels are significantly higher in patients that do not show a MTX response after 12 weeks [100]. Finally, a *post hoc* analysis of serum samples from the Swedisch Farmacotherapy (SWEFOT) trial demonstrated a poor response to MTX monotherapy in patients that are positive for survivin [101], a proto-oncogene that has also been shown to be upregulated in the serum of preclinical phase RA-patients [102]. Overall, a number of promising theranostic markers for MTX response exist, but these generally have only been tested a limited numbers of patients and await validation in larger independent patient cohorts. Therefore, the need for novel clinically applicable biomarkers that can predict MTX response before the onset of treatment remains high. Discovery of novel, more sophisticated theranostic biomarkers and their incorporation in the current diagnostic, prognostic and theranostic models could result in an improvement of the global quality of RA care, reducing costs for extra medication, (para-) medical consultations and hospitalizations to deal with insufficient disease control, not to forget its consequences in terms of adverse events, comorbidity, psychosocial wellbeing, orthopedic surgery need and vocational/professional participation [56] .

Screening for novel theranostic autoantibody markers using serological antigen selection

As mentioned above, there is an urgent need for novel theranostic biomarkers in daily clinical practice since there is evidence that the traditional serological markers for RA (RF and ACPA) do not perform well enough for the stratification of patients with RA, especially in view of treatment allocation [85]. To be relevant for clinical practice the discovery of new serological biomarkers should ideally be based on the analysis of samples derived from a well-defined and representative patient population that is followed prospectively with regular documentation of disease activity, functionality and joint damage [92]. Patients in such a cohort should

preferentially be treated in a highly standardized way and according to the current treatment paradigm based on rapid remission induction followed by a treat to target approach, certainly for the discovery of novel prognostic and theranostic biomarkers. In the CareRA trial, a pragmatic randomized controlled trial involving 400 early RA patients, both high-risk as well as low-risk patients based on the current prognostic standards, were treated according to a strict protocol reflecting current international treatment recommendations [85,86] (Figure 1). Throughout the trial, an extensive clinical dataset and serum samples at baseline and follow up samples throughout a two year period after treatment initiation, were collected. Such a collection of well-defined serum samples will allow us to probe for different autoantibody profiles using serological antigen selection (SAS), a powerful high-throughput screening technology to identify novel autoantibody targets using cDNA phage display. Of particular interest will be the use of baseline samples to find markers that predict positive or negative response to the Cobra treatment regimens, while the use of intermediary samples can be used to find novel markers that predict disease relapse in patients that achieved early remission.

1 cDNA phage display

Phage display was first described 30 years ago by George Smith [103] and is now a commonly used technique that uses the expression of exogenous proteins or peptides on the surface of phage particles to probe for protein-protein interactions (reviewed in [104,105]). Different types of phage are used for phage display including the filamentous M13 phage, the temperate λ phage and the lytic T7 phage. One of the main strengths of phage display is the use of large diversity phage libraries, which can contain 10^6-10^7 independent clones for cDNA libraries [106-108] and up to 10^{10} clones for random or overlapping peptide libraries. This increases the chance to find relevant, high-affinity interacting proteins, which is required for applications like epitope mapping of antibodies. Furthermore, the direct link between the

protein expressed on the phage surface and the genetic material inside the phage particle allows sequential rounds of target selection and amplification and easy target identification by DNA sequencing.

Filamentous phage can only infect bacteria expressing F-pili to which they bind for entering the cell. Upon entry, the phage uses the bacterial machinery for replicating its circular genome and synthesizing proteins required for phage packaging. Assembly and release of new phage particles occurs at the bacterial plasma membrane and does not involve cell lysis. The M13 filamentous phage consists of a circular single stranded DNA genome surrounded by a protein coat composed of five different proteins (reviewed in [105]). The protein coat has a rod-like, elongated shape with several thousand copies of the major coat protein pVIII covering the length of the phage, while the two ends of the filament are capped 3 to 5 copies of each of the minor coat proteins pIII, pVI, pVII and pIX. Phage display libraries have been made using each of the five coat proteins, but fusion to the minor coat proteins pIII and the major coat protein pVIII are most commonly used [109]. In most phage display systems, the fusion proteins are expressed from phagemid vectors. These carry a bacterial and phage origin of replication, a phage packaging signal, an antibiotic resistance gene and the phage coat protein that will carry the fusion [110]. The other proteins required for phagemid replication and packaging are provided by superinfection with a helper phage. To ensure that the phagemid is preferentially packed in the secreted phage particles, replication deficient helper phage like M13KO7 is often used [111].

Fusion to pIII and pVIII is almost exclusively to the N-terminus. This orientation does not allow cloning of full length cDNA since the endogenous stop codon will prevent formation of a fusion protein. However, cDNA synthesis using internal priming with random hexameric

primers or fragmentation of the cDNA molecules can generate cDNA fragments that lack the natural stop codon, allowing N-terminal fusion. Such a cDNA library can be further enriched for in-frame clones using the so-called Hyperphage [112,113]. This is a helper phage that lacks a wild type pIII gene, so functional phage particles can only be formed if the cDNA fragment is cloned in frame with the downstream gene III. Display of a cDNA library on pIII can also be achieved using the indirect Jun-Fos system [114]. This system relies on the strong association of Jun, which has the library inserts cloned to its C-terminus, and its interaction partner Fos, fused to pIII.

On the other hand, since pVI is the only phage coat protein with its C-terminus facing outward, it is highly suitable for direct cloning of full length cDNA fusion libraries. Since phage display using pVI fusion is monovalent, generating phage particles with one fusion protein per phage particle [115], it allows for the identification of high-affinity interactions. This display method has been successfully used for the isolation of serine protease inhibitors from a hookworm cDNA library [115], and a series of reports from our research group describe the construction and use of patient- and tissue-specific pVI cDNA libraries expressing colorectal cancer tumor antigens [116] and autoantigens of the autoimmune diseases RA [107], MS [22,117] and clinically isolated syndrome [118]. Recently, our research group has also reported on the construction of a novel human spinal cord cDNA phage display library [119].

2 Serological antigen selection

The autoantibody profile of a specific patient (sub)population can be determined using a method called serological antigen selection (SAS). Here a high diversity disease- or tissue-specific cDNA phage display library is used to identify the antigenic targets that elicit an antibody response (Figure 2). Depending on the application the antibody markers will be used for, patient

sera of a specific (autoimmune) disease or a specific subpopulation, such as patients with early diagnosis, poor prognosis or positive or negative response to a particular treatment regimen, can be chosen. These patients should represent the general characteristics of the entire patient population including age, gender and possible genetic markers. Usually 5 to 10 patient sera are selected and pooled for the panning procedure. SAS has been successfully used to identify novel candidates in the antigenic repertoire of colorectal cancer [116], MS [22,120,117], CIS [118,121], RA [50] and spinal cord injury (our unpublished data). The cDNA phage display libraries used in these studies contain a primary diversity in the range of 10^6-10^7 bacterial phagemid clones [106-108]. This bacterial library is infected with M13KO7 helper phage and phage particles displaying the antigenic repertoire are assembled and released in large numbers. This phage library is panned against the autoantibodies present in the sera of a selection of patients of interest. Pooled patient serum is incubated with the phage library in solution allowing complex formation between the displayed human proteins and serum autoantibodies. After immunoprecipitation of these complexes, bound phage is eluted and amplified by re-infecting pilus-bearing bacteria. Multiple rounds of this positive selection are repeated in order to amplify the specific binding variants. Non-specific binders are eliminated in a final negative selection round where those antigens that bind to antibodies from age- and gender-matched healthy control sera, are removed. Finally, the resulting antigenic profile can be determined using DNA fingerprinting and sequencing of the cDNA inserts cloned behind gene VI.

The use of a human cDNA phage display library derived from disease-specific tissue to discover novel autoantigens offers several advantages compared with the use of human protein arrays. Since a cDNA library covers the transcriptome of the tissue it is isolated from, it contains autoantigens that are specifically upregulated in disease-related tissues or cell types isolated from patients with active disease. This has been shown in anti-neutrophil cytoplasmic antibody

(ANCA) positive vasculitis patients, where the expression of the autoantigens proteinase3 and myeloperoxidase was specifically upregulated in neutrophils [122]. Comparison of cDNA libraries from MS patients and healthy controls showed a differential upregulation of several described autoantigens in the MS patient-derived cDNA library [123]. Furthermore, patient material allows for the isolation of disease-specific sequence variants while protein arrays are usually limited to the canonical gene sequences. Glucose-6-phosphate isomerase (GPI) [124] and heterogeneous nuclear ribonucleoprotein complex A2 (hnRNP-A2) [125] autoantibodies have been reported in several arthritic diseases including RA. Sequence variants that lead to partial deletions of the GPI gene [126] and hnRNPA2/B1 splice variants that lack exon 9 [127] are more antigenic and induce a more pronounced autoantibody response. Finally, cDNA phage display libraries are often cloned in a phagemid that uses cloning in three different reading frames to allow the cDNA to be expressed in-frame with the phage coat protein in one out of three clones [106]. Therefore, two thirds of the library consists of out of frame fusions that generate random peptide sequences. This results in a phage display library that combines the expression of proteins and peptides that respectively allow the isolation of conformational epitopes and linear epitopes or mimotopes [128,129].

3 A well-defined patient population is required for SAS

In order to identify novel autoantibody markers with theranostic value, we can rely on serum samples from the well characterized CareRA patient cohort. The CareRA study is a broad research project anchored to a pragmatic multicenter randomized controlled trial in collaboration with 12 rheumatology practices and almost 400 participating patients [86]. Patients were stratified to risk groups based on classical prognostic factors (RF/ACPA, disease activity and erosions) (Figure 1). High-risk patients (n=300) were randomized to one of three treatment arms consisting of different Cobra variants (classic/avant-garde/slim) and low-risk

patients (n=100) either to a step-up from MTX monotherapy or a step-down Cobra slim therapy. Treatment in all arms was adjusted if needed to a target of low disease activity. From the CareRA trial we learned that all Cobra regimens resulted in very high remission rates (70%), that combinations of csDMARDs (Cobra avant-garde and classic) were not superior to MTX alone (Cobra slim) when given together with a step-down GC regimen, that high initial prednisone dosages (60 mg) were not superior to moderate dosages (30 mg) for remission induction and that Cobra slim showed less adverse events than csDMARD combinations in high-risk early RA [86]. In low-risk RA patients Cobra slim resulted in a shorter time to remission without more adverse events compared to step-up therapy [85]. Based on these results from the CareRA trial, applying the treat to target principle and pending better risk stratification, Cobra slim can be proposed as an effective one-size-fits-all initial treatment solution for all RA patients and a benchmark for future cost-effectiveness trials with novel therapeutics.

Starting from this sample collection there are many opportunities for the discovery and validation of novel biomarkers for diagnosis and classification, but also for theranostic purposes. Today however, for us the most urgent unmet need in the clinic is a tool for rapid identification of early RA patients at risk of an insufficient response to a combination of csDMARDs combined with GCs, like the Cobra Slim regimen, which we consider the gold standard [85,86]. There is ample evidence that the response during the first weeks after treatment initiation is predictive of the disease course thereafter, more or less irrespective of the initial treatment choice [56]. Therefore it should be possible to discover and validate biomarkers predictive of the response to treatment according to the present standards by comparing baseline serum samples from CareRA patients achieving remission or not, after 16 weeks of treatment.

4 Construction of a novel RA cDNA phage display library

We have constructed a cDNA phage display library that represents the transcripts expressed in human knee synovial tissue (Figure 3). Synovium was obtained from three female RA patients that have undergone knee replacement surgery. Since there might be interpersonal variation with respect to the expressed antigens, the incorporation of synovial material from multiple patients increases the chance to find relevant antigenic sequences. Total RNA was extracted and purified from synovial tissue of the different RA patients. RNA integrity was confirmed on a 2100 Bioanalyzer (Agilent) using an RNA Nano chip and the Eukaryote Total RNA Nano program. Messenger RNA (mRNA) expressed in the tissues was converted into cDNA using an oligo (dT)22 primer containing a XhoI restriction site (Maxima H-minus double stranded cDNA synthesis kit, Thermo Fisher). The Maxima H-minus reverse transcriptase lacks RnaseH activity, and is more stable at higher temperatures resulting in a higher processivity and a higher chance to cover the entire open reading frame, starting from the poly-A tail. The oligo(dT) primer used, was modified with a VN anchor at the 3' end, where V stands for dA, dC, or dG and N for dA, dC, dG or dT. Such an anchored oligo(dT) primer only allows primer annealing at the very 5' end of the poly-A tail, leading to reduced poly-A slippage and a more efficient cDNA synthesis [130]. Each of the three pools of blunt-ended, double-stranded cDNA of the different tissue donors, was then ligated with its unique adaptor. These consisted of a free EcoRI overhang, a NotI restriction site and either a SalI, AgeI or ClaI restriction site. The difference in sequence between the latter three enzyme recognition sites can be used as a molecular barcode afterwards to identify the donor tissue the cDNA originated from. The total cDNA mixture was size-fractionated using agarose gel electrophoresis and cDNA inserts ranging from 200 to 10,000 bp were directionally cloned in the pSPVI-A, pSPVI-B and pSPVI-C phagemid vectors using the EcoRI an XhoI restriction enzymes. Each pSPVI vector has a different reading frame, which allows cloning of the cDNA insert in frame with the M13 phage

gene VI in one of the three vectors [106]. The protein encoded by the human cDNA is then expressed as a C-terminal fusion to the M13 minor coat protein pVI. The entire cDNA library was electroporated to F pilus-expressing TG1 *Escherichia coli* cells resulting in a total primary diversity of 9,0 x 10^6 bacterial colonies.

The quality of our primary cDNA phage display library was investigated by sequencing the inserts cloned behind gene VI of 70 randomly selected clones. A good distribution between each of the three reading frames of the pSPVI-A, pSPVI-B and pSPVI-C phagemid vectors was found (Figure 4a). Twelve clones (17,1%) contained no insert leading to a slight decrease in our true library diversity. After correction for the empty pSPVI clones, we still have a diversity of 7,47 x 10^6 insert containing clones. Since this library contains the cDNA inserts of three different tissue donors, cloned into three different reading frames, each donor has the potential for 0,83 x 10^6 clones that are in frame with the M13 gene VI. With an estimated 19,000 to 20,500 protein coding genes in the human genome [131,132], each gene approximately has a 40-fold coverage on average. With such a large library diversity, also genes with lower expression levels in the synovium are likely to be represented in our library.

Each of the 58 remaining insert-containing clones could be traced to one of the three tissue donors using our patient-specific adaptors, showing that each donor was equally represented in the entire library (Figure 4b). Inserts were compared to human reference mRNA or genomic sequences using the Basic Local Alignment Search Tool (BLAST) on the NCBI website (www.ncbi.nlm.nih.gov/BLAST) (Table 1). This allowed the identification of the mRNA the insert originated from and the region of the transcript that was fused to gene VI (Figure 4c). Of the insert containing clones, 21 contained a sequence starting in the 5' untranslated region (UTR) of the cDNA (36,2 %), 16 in the coding region (27,6 %) and 12 in the 3' UTR (20,7).

For 9 clones, the insert did not originate from mRNA but from ribosomal RNA (rRNA) (5 clones, 8,6%) or genomic DNA (4 clones, 6,9%).

In a large proportion of clones, the cDNA insert was cloned in frame with the M13 gene VI (Table 1). This was the case for 42,9 % of the 5'UTR fusions and 50% of the fusions to the coding region. The clones that had an in-frame fusion to the 5'UTR contained part of the translated 5'UTR followed by the full length protein, which could range in size between 91 and 685 amino acids (aa). In-frame fusions to the coding region could be found in different parts of the open reading frame, resulting in fusion proteins with a size between 10 and 354 aa. The remaining clones contained an out of frame fusion or fusion to a non-coding region, resulting in fusion constructs with a random peptide sequence between 1 and 147 aa. Approximately one in two (26/49, 53,1%) of the cDNA clones encoded for proteins with a cytoplasmic localization such as the generally expressed beta actin (ACTB), alpha 1 tubulin (TUBA1C) and several ribosomal protein genes. Genes encoding ribosomal protein genes represented the largest fraction of our sample (13/49, 26,5%). Subunits of ferritin (FTH1, FTL), a cytoplasmic protein involved in iron storage, have been isolated four times, originating from two different tissue donors. Ferritin has been found to be specifically upregulated in RA synovium [133,134]. Also of interest is the expression of S100A9, a subunit of the cytoplasmic calprotectin protein complex. Calprotectin is a calcium-binding complex mainly expressed in macrophages and neutrophils. Under a number of inflammatory conditions such as RA, Crohn's disease and MS, it is also released into the extracellular environment where it acts as danger associated molecular pattern (DAMP) (reviewed in [135]). In RA patients, its elevated levels in both serum and synovial fluid correlate with the degree of inflammation and joint damage [136]. Approximately 18% (9/49) of the insert containing clones encoded extracellular proteins such as the extracellular matrix (ECM) proteins collagen type I alpha 1 (COL1A1) and fibronectin 1 (FN1)

and the ECM protein-modifying enzymes galactosidase beta 1 (GLB1) and lysyl oxidase-like 1 (LOXL1). Additional extracellular proteins of interest were the proteases HtrA serine peptidase 1 (HTRA1) and Cathepsin B (CTSB) and protease inhibitors serpin peptidase inhibitor, clade F (SERPINF1) and clade G (SERPING1). HtrA1 and CtsB are secreted by RA fibroblast-like synoviocytes (FLS) and contribute respectively to extracellular matrix degradation and FLS migration and invasion [137-139]. SERPINF1 mutants have been shown to lead to osteogenesis imperfecta, a connective tissue disorder characterized by low bone mass and bone fragility [140] while SerpinG1 is responsible for inhibition of the C1 component of the classical complement system [141]. Three clones encoded proteins with a plasma membrane localization, such as HLA class II histocompatibility antigen gamma chain (CD74), which is involved in antigen processing [142] and required for osteoclastogenesis induced by its ligand macrophage migration inhibitory factor (MIF) [143,144]. Finally, cDNA products were found that localize to the nucleus (10,2%), endoplasmic reticulum (6,1%), Golgi (4,1%) and one gene with unknown function and localization.

Future perspectives

Our newly constructed cDNA phage display library is a high diversity representation of the genes expressed in synovia of three different RA patients. A large proportion of this library consists of full length genes and gene fragments cloned in frame with the M13 gene VI. Therefore, this library is a tool with great potential to discover novel autoantigens for RA. The combination of this library with selected pooled sera has the potential to uncover novel autoantibody biomarkers for very different applications. The use of samples from the CareRA patient cohort will allow us to isolate novel theranostic markers in baseline sera that predict early response to the Cobra regimens, prognostic markers in intermediary sera that predict disease relapse, but also novel diagnostic markers that would identify seronegative patients.

Ethical approval: “All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.”

Informed consent: “Informed consent was obtained from all individual participants included in the study.”

1. Abbreviations

Rheumatoid arthritis (RA)

systemic sclerosis (SSc)

systemic lupus erythematosus (SLE)

anti-citrullinated protein antibodies (ACPA)

collagen antibody-induced arthritis (CAIA)

Non-steroidal anti-inflammatory drugs (NSAIDs)

disease-modifying anti-rheumatic drugs (DMARDs)

tumor necrosis factor α (TNF-α),

rheumatoid factor (RF)

cyclic citrullinated peptides (CCP)

undifferentiated arthritis (UA)

anti-carbamylated protein (anti-CarP)

anti-neutrophil cytoplasmic antibody (ANCA)

untranslated region (UTR)

2. Tables

Table1: sequences identified in RA cDNA library

NCBI code	cDNA identity	fusion type	size fused protein (aa)	in frame pVI
NM_000404.3	galactosidase beta 1 (GLB1)	5'UTR	685	Y
NM_000981.3	ribosomal protein L19 (RPL19)	5'UTR	376	Y
NM_002356.6	myristoylated alanine rich protein kinase C substrate (MARCKS)	5'UTR	356	Y
NM_153649.3	tropomyosin 3 (TPM3), variant 1 (TPM3)	5'UTR	266	Y
NM_002032.2	ferritin, heavy polypeptide 1 (FTH1)	5'UTR	243	Y
NM_000146.3	ferritin, light polypeptide (FTL)	5'UTR	238	Y
NM_182491.2	zinc finger, AN1-type domain 2A (ZFAND2A)	5'UTR	234	Y
NM_001017.2	ribosomal protein S13 (RPS13)	5'UTR	155	Y
NM_001024.3	ribosomal protein S21 (RPS21)	5'UTR	91	Y
NM_002032.2	ferritin, heavy polypeptide 1 (FTH1)	5'UTR	103	N
NM_005576.3	lysyl oxidase-like 1 (LOXL1)	5'UTR	41	N
NM_000992.2	ribosomal protein L29 (RPL29)	5'UTR	37	N
NM_001016.3	ribosomal protein S12 (RPS12)	5'UTR	37	N
NM_006423.2	Rab acceptor 1 (prenylated) (RABAC1)	5'UTR	34	N
NM_002933.4	ribonuclease A family member 1 (RNASE1)	5'UTR	34	N
NM_021104.1	ribosomal protein L41 (RPL41)	5'UTR	34	N
NM_000088.3	collagen type I alpha 1 (COL1A1)	5'UTR	14	N
NM_015683.1	arrestin domain containing 2 (ARRDC2)	5'UTR	12	N
NM_012423.3	ribosomal protein L13a (RPL13A)	5'UTR	11	N
NM_153741.1	dolichyl-phosphate mannosyltransferase polypeptide 3 (DPM3)	5'UTR	8	N
NM_002948.3	ribosomal protein L15 (RPL15)	5'UTR	5	N
NM_212478.2	fibronectin 1 (FN1)	coding	354	Y
NM_006600.3	nuclear distribution protein (NUDC)	coding	322	Y
NM_031369.2	heterogeneous nuclear ribonucleoprotein D (HNRNPD)	coding	269	Y
NM_000975.3	ribosomal protein L11 (RPL11)	coding	178	Y
NM_022551.2	ribosomal protein S18 (RPS18)	coding	149	Y
NM_001357.4	DEAH-box helicase 9 (DHX9)	coding	125	Y
NM_000177.4	Gelsolin (GSN)	coding	43	Y
NM_005103.4	fasciculation and elongation protein zeta 1 (FEZ1)	coding	10	Y
NM_000062.2	serpin peptidase inhibitor, clade G (SERPING1)	coding	100	N
NM_001015.4	ribosomal protein S11 (RPS11)	coding	52	N
NM_002965.3	S100 calcium binding protein A9 (S100A9)	coding	30	N
NM_001101.3	actin, beta (ACTB)	coding	24	N
NM_002775.4	HtrA serine peptidase 1 (HTRA1)	coding	14	N
NM_001015.4	ribosomal protein S11 (RPS11)	coding	7	N
NM_001303114.1	tubulin alpha 1c (TUBA1C)	coding	1	N
NM_001011.3	ribosomal protein S7 (RPS7)	coding	1	N

NM_023080.2	C8orf33 (C8orf33)	3'UTR	65	N/A
NM_001908.4	Cathepsin B (CTSB)	3'UTR	53	N/A
NM_002032.2	ferritin, heavy polypeptide 1 (FTH1)	3'UTR	42	N/A
NM_014045.3	low density lipoprotein receptor-related protein 10 (LRP10)	3'UTR	27	N/A
NM_001025159.2	major histocompatibility complex, class II invariant chain (CD74)	3'UTR	14	N/A
NM_001101.3	actin, beta (ACTB)	3'UTR	14	N/A
NM_182547.3	transmembrane p24 trafficking protein 4 (TMED4)	3'UTR	10	N/A
NM_002615.5	serpin peptidase inhibitor, clade F (SERPINF1)	3'UTR	8	N/A
NM_014604.3	(human T-cell leukemia virus type I) binding protein 3 (TAX1BP3)	3'UTR	5	N/A
NM_207012.2	adaptor related protein complex 3 mu 1 subunit (AP3M1)	3'UTR	3	N/A
NM_173179.3	solute carrier family 35 (GDP-fucose transporter), member C2 (SLC35C2)	3'UTR	2	N/A
NM_015360.4	Ski2 like RNA helicase 2 (SKIV2L2)	3'UTR	1	N/A
NR_046235.1	45S pre-ribosomal 5 (RNA45S5)	rRNA	132	N/A
NR_046235.1	45S pre-ribosomal 5 (RNA45S6)	rRNA	56	N/A
NR_046235.1	45S pre-ribosomal 5 (RNA45S7)	rRNA	45	N/A
NR_046235.1	45S pre-ribosomal 5 (RNA45S8)	rRNA	41	N/A
NR_003286.2	18S ribosomal 5 (RNA18S5)	rRNA	0	N/A
	(genomic)	genomic	147	N/A
	(genomic)	genomic	15	N/A
	(genomic)	genomic	6	N/A
	(genomic)	genomic	4	N/A

3. Figure legends

Figure 1 Patient stratification and randomization in the CareRA trial

All patients that participated in the CareRA trial (n=380) were risk stratified according to the classical prognostic factors, RF/ACPA status, disease activity and erosions. The high risk patients (n=290) were randomized into three groups: Cobra classic (MTX + sulfasalazine + 60 mg prednisone tapered to 7,5 mg daily from week 7), Cobra slim (MTX + 30 mg prednisone tapered to 5 mg daily from week 6) and Cobra avant-garde (MTX + leflunomide + 30 mg

prednisone tapered to 5 mg daily from week 6). The low risk patients (n=90) were randomized into two groups: tight step-up (MTX monotherapy) and Cobra slim. MTX, methotrexate; mg, milligram; RF, rheumatoid factor; ACPA, anti-citrullinated protein antibodies.

Figure 2 Identification of antigenic targets via Serological antigen selection (SAS)

The RA cDNA phage display library is incubated with pooled RA serum, allowing the formation of antigen-antibody complexes between IgG in the RA serum and their target antigens displayed on the surface of the phage. These complexes are captured on a solid surface using anti-human IgG antibodies, followed by washing away the non-bound phage particles. Retained phage particles are eluted, amplified and used for a new round of positive selection with RA-serum. After repeating positive selection 3 to 4 times, the eluted fraction is depleted of aspecific antibody reactivity by one round of negative selection. Here, phage is incubated with an age- and gender-matched healthy control serum pool, allowing the formation of antigen-antibody complexes between healthy control IgG and their target antigens on the phage. After this negative-affinity selection, nonbound phage particles are isolated and cDNA inserts are amplified with PCR and characterized with DNA fingerprinting and sequencing.

Figure 3 Construction of a human RA cDNA phage display library

Total RNA was independently isolated from knee synovial tissue of three different RA patients. The poly(A) mRNA was converted to first strand cDNA using a VN-anchored oligo d(T)22 primer that contained a XhoI restriction site at its 5' end. After second strand synthesis, three different adaptors were ligated to each of the blunt-end ds cDNA mixtures of the three RA patients. Each of the adaptors contained a free EcoRI overhang, used for cloning to the pSPVI vector, and either a SalI (RA-patient1), AgeI (RA-patient2) or ClaI (RA-patient3) restriction site, used for identification. The resulting cDNA molecules were size-fractionated and cloned

simultaneously into the pSPVI-A, pSPVI-B and pSPVI-C phagemid vectors using EcoRI and XhoI, allowing expression of the cDNA fragments in three reading frames behind gene VI. The cDNA library was inserted in TG1 *E.coli* cells using electroporation. Phage particles displaying the human cDNA-encoded proteins were assembled and released after superinfection of TG1 cells with M13KO7 helper phage. ds, double stranded; X, XhoI; E, EcoRI, S, SalI; A, AgeI; C, ClaI; V, dA, dG or dC; N, dT, dA, dG or dC.

Figure 4 Characterization of the cDNA library clones

From a representative sample of the entire cDNA library, the inserts of 70 randomly selected bacterial clones have been characterized. The insert was amplified via PCR using general primers in the regions flanking the cDNA insert and the sequence was determined starting from gene VI. (a) The region directly behind gene VI determines the reading frame of the cDNA insert. pSPVI-B and pSPVI-C respectively have one and two extra basepairs in this region, compared to the pSPVI-A vector. The reading frame was determined of all 70 clones, including the clones containing empty pSPVI vector. (b) Each insert-containing clone (n=58) could be traced back to one of the three synovial tissue donors using our RA-patient-specific adaptors. (c) For each insert-containing clone (n=58), the region of the cDNA transcript that was fused to gene VI was determined. cDNA inserts originating from mRNA transcripts could be fused at their 5'UTR, coding region or 3'UTR. Inserts not originating from mRNA, but form rRNA or genomic DNA were also retrieved. UTR, untranslated region; mRNA, messenger RNA; rRNA, ribosomal RNA

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