D6.8 Compendium on current researches for inducing clinical therapeutic tolerance

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Rheuma Tolerance for Cure

WP6 – Clinical trials

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Publishable Summary

Compendium on current researches for inducing clinical therapeutic tolerance.

With one of the main aims of the research taking place in our RTCure consortium being to better understand, and to achieve, therapeutic tolerance in Rheumatoid Arthritis (RA), we decided to write a series of reviews on the current state of the topic. We have several experts in RTCure and have divided the topic into the following chapters:

1. *Previous and current attempts to induce clinical therapeutic tolerance – with a focus on RA but including other autoimmune, allergic and transplant settings*
2. *Postulated autoantigens and supporting data in RA*
3. *Information on the 'pre-RA' state, including data on B and T cell epitope spreading involving key autoantigens, illustrating how tolerance is lost over time.*
4. *Potential biomarkers of the tolerant state*

To maximise impact we had our reviews commissioned for publication as a series of articles in Lancet Rheumatology. All four have now been written and published.

Results

Four published reviews on the topic of therapeutic tolerance induction. The author accepted versions of the articles can be found below.

Tolerance-inducing medicines in autoimmunity: rheumatology and beyond. James A Stanway, John D Isaacs. Published: September, 2020. The Lancet Rheumatology Vol. 2 No. 9e565–e575. DOI: https://doi.org/10.1016/S2665-9913(20)30100-4

Autoantigens in rheumatoid arthritis and the potential for antigen-specific tolerising immunotherapy. Hendrik J Nel, Vivianne Malmström, David C Wraith, Ranjeny Thomas. Published: November, 2020. The Lancet Rheumatology. Vol. 2 No. 11e712–e723. DOI: https://doi.org/10.1016/S2665-9913(20)30344-1

The autoimmune response as a potential target for tolerance induction before the development of rheumatoid arthritis. Rene EM Toes, Karim Raza. Published: March, 2021. The Lancet Rheumatology. Vol. 3. No. 3e214–e223. DOI: https://doi.org/10.1016/S2665-9913(20)30445-8

Biomarkers of tolerance in immune-mediated inflammatory diseases: a new era in clinical management? Kenneth F Baker, Jasmine P X Sim, John D Isaacs. Published: May, 2021 The Lancet Rheumatology Vol. 3 No. 5e371–e382. DOI: https://doi.org/10.1016/S2665-9913(21)00069-2

Tolerance Inducing Medicines in Autoimmunity: Rheumatology and Beyond

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Abstract:

Autoimmunity is managed currently with generalised immunosuppression, which is associated with serious side effects including infection and cancer. An ideal treatment strategy would be to induce 'immune tolerance', reprogramming the immune system to cease recognising self as a threat. Drug free remission should follow such an intervention, representing a paradigm shift in the treatment of autoimmune disease. Tolerance induction is achievable in animal models of autoimmunity but translation to the clinic has been slow. Nonetheless, recent progress has been encouraging, including restoration of therapeutic responsiveness, and drug free remission, achieved with stem cell transplantation in refractory autoimmunity; and significantly delayed onset of type 1 diabetes in high-risk individuals following a brief intervention with anti-CD3 monoclonal antibody. Looking forward, antigen-specific interventions should provide highly targeted, personalised medicine, avoiding generalised immunosuppression entirely. Such trials have already commenced, using both direct autoantigenic peptide administration as well as cellular therapies and other vehicles as carriers. Here we review the history of immune tolerance induction, focussing on rheumatological disease but also highlighting essential data from other specialties. We highlight key unanswered questions, which will be covered in forthcoming reviews in this series.

Introduction

Our immune system protects us from pathogens and cancer. Key to its function is the ability to distinguish 'self' from 'non-self', known as immune tolerance (figure 1). Malfunction of tolerance can lead to autoimmune diseases such as rheumatoid arthritis (RA), multiple sclerosis (MS) and type 1 diabetes (T1D). Ever since the first demonstration that tolerance could be manipulated artificially[1], clinicians and scientists have worked to identify methods to achieve 'therapeutic tolerance', to treat autoimmunity, allergy and transplant rejection. During this time our understanding of human immunology has transformed from its earliest stages as a phenomenology to the present day, where legions of cell types and a vast array of molecular mediators are described, both have formed the targets of tolerogenic endeavours. As the field of human immunology has developed, approaches to tolerance induction have become more sophisticated, beginning with non-specific lymphocyte targeted monoclonal antibodies (mAbs) in the late 1980s to the antigen-specific peptides and cellular therapies of today.

Why Induce Tolerance and How to Define it?

Whilst the treatment of autoimmune and inflammatory disease has been transformed by advances in immunosuppression and the development of mAbs, current treatments are still suboptimal as they depend on generalised immunosuppression. Autoimmunity, however, starts with a small population(s) of self-reactive lymphocytes misbehaving, the vast majority of the lymphocyte population remaining innocent and reacting appropriately to potential threats. Generalised immunosuppression does not discriminate between healthy and pathologic cells, leading to significant collateral damage and risk of infection and cancer. Furthermore, none of these immunosuppressive and anti-inflammatory therapies can 'cure' autoimmune disease, which usually relapses on withdrawal of treatment.

Therapeutic tolerance induction is the Holy Grail in the treatment of autoimmunity, transplantation and allergy. In transplantation, tolerance can be defined as graft acceptance in the absence of continued immunosuppression and in allergy as the ability to encounter antigen without developing hypersensitivity. In autoimmunity, the equivalent should be cure, or prevention of disease onset in those at risk. However, these outcomes may be ambiguous in diseases that naturally relapse and remit. For how long must disease onset be delayed to define prevention? How long must drug free remission last to achieve cure? Whereas life-long tolerance induction is desirable, infrequent interventions interspersed with periods of drug free remission may

be preferable to current treatment paradigms. Lastly, whilst clearly not equivalent to tolerance induction, the restoration of therapeutic responsiveness in refractory disease may be a useful outcome.

When to induce tolerance

Tolerance induction can be considered at different points in the natural history of autoimmunity (figure 2). At the earliest, tolerogenic interventions may be employed in high risk individuals to prevent disease onset. It is widely accepted that tolerance is broken many years before symptoms develop and trials of prevention are already emerging in T1D and RA. Treating asymptomatic individuals, however, requires careful consideration of risk. A potent, immunosuppressive intervention could be justified in an individual who was at high risk of developing a serious and potentially fatal autoimmune condition with limited treatment options such as diffuse scleroderma. Justification would be more difficult for a condition such as RA, which is arguably less serious and for which effective (though not curative) treatments exist. A major unmet need is immune biomarker(s) that distinguish the tolerant from the autoimmune state and which can be used to monitor the effects of tolerogenic interventions. Such biomarkers are especially important in the preventative setting, to provide an efficacy measure in an asymptomatic population.

If prevention cannot be justified, tolerance induction could be attempted at disease onset. In this scenario, a more aggressive intervention may be justified to avert life-long illness associated with premature mortality as in RA or T1D. At this stage, tolerogenic treatments may need to be administered alongside or following a course of traditional disease modifying therapy – favourable immune modulation may develop slowly with a tolerogenic therapy, which will not necessarily suppress inflammation in the short-term. Biomarkers are still required at this stage, to identify that treatment is working, and possibly to identify if and when traditional treatment may be stopped.

Tolerance induction may play less of a role in established disease. As autoimmunity progresses, the immune response to self diversifies in a phenomenon known as ‘epitope spreading’. This may render therapeutic tolerance induction (especially antigen specific modalities) more difficult to achieve. Furthermore, the symptom burden now reflects irreversible damage in addition to inflammation and, whilst tolerance induction may remain a desirable outcome, it is unlikely to restore normality. Returning to the risk:benefit equation, however, it may still be considered worthwhile to attempt tolerance induction if disease has proved refractory to conventional treatments (or conventional treatments do not exist).

As with all other aspects of medicine, the potential benefits of any tolerogenic intervention need to be balanced against the risks. The need for safety is increased in preventative strategies, particularly where disease development is not certain. Higher risk interventions may be justifiable where disease is established or inevitable, particularly where conventional treatment options are limited and disease outcomes poor. Similarly, it may be harder to justify an aggressive approach in children, although children have their disease for more years than adults.

Broadly speaking, there are two approaches to therapeutic tolerance induction, antigen specific and antigen non-specific. In the former, therapy targets only disease-associated autoreactive lymphocytes. In contrast, antigen non-specific approaches impact on all adaptive (and sometimes innate) immune responses. In contrast to conventional immunosuppression, however, if tolerance is induced treatment can be discontinued. Antigen specific approaches are clearly more desirable, particularly for disease prevention or perhaps in children. They currently represent a significant technical challenge, however, particularly in terms of choosing the most appropriate autoantigens (or autoreactive lymphocytes) to target in a particular individual. Non-specific approaches are currently more tractable but may induce at least temporary immunosuppression, necessitating careful consideration of risk.

Here we will review tolerogenic strategies that have been studied in autoimmunity and those that are likely to emerge in the years ahead. Whilst our discussion focusses on rheumatic disease, a true overview of the field also requires reference to other disciplines.

Non antigen specific tolerance induction

Haematopoietic Stem Cell Transplantation

Haematopoietic stem cell transplantation (HSCT) is used for the treatment of conditions including lymphoma, leukaemia and immunodeficiency. The aim of HSCT is to replace a malfunctioning immune system with a new one generated from immature precursor cells (figure 3). This is achieved by high doses of cytotoxic chemotherapy or radiation to eradicate the diseased immune system (myeloablation) followed by infusion of haematopoietic stem cells to regenerate a new immune system. Stem cells can be derived from the patient (autologous HSCT) or from a matched donor (allogeneic HSCT). Allogeneic HSCT carries a risk of fatal graft versus host disease, for this reason, autologous HSCT is by far the more commonly employed method in autoimmunity.

With its ability to replace a diseased immune system, HSCT has tolerogenic potential. In the field of rheumatology, systemic sclerosis (SSc) has received the most attention, with three randomised trials now complete. The ASSIST trial reported in 2011, 8 of 10 patients randomly assigned to receive HSCT showed sustained improvements in modified Rodnan skin score (mRSS) and forced vital capacity (FVC) compared to none of nine cyclophosphamide treated controls, over an average follow up of 2.6 years[2]. In 2014, the phase III ASTIS study compared HSCT to cyclophosphamide in 156 early diffuse scleroderma patients. Whilst HSCT produced a significant increase in event free survival during an average follow up of 5.8 years, this was accompanied by an increase in early treatment related mortality (10% vs 0%). HSCT led to improvements in other disease metrics: mRSS improved by 19.9 points compared to 8.8 with cyclophosphamide; total lung capacity improved by 5.1% compared to a reduction in controls of 1.6%[3]. In 2018 the SCOT trial, using adjunctive total body irradiation for myeloablation, provided further evidence for efficacy of HSCT but with lower treatment related mortality than reported in ASTIS. Of note, at 54 months only 9% of transplanted patients had initiated DMARDs compared to 44% in the cyclophosphamide control group[4]. Given the fibrotic nature of the disease not all symptoms and complications resolve; nonetheless, the lack of progression despite the absence of DMARDs suggests that HSCT has tolerogenic action. EULAR now recommend consideration of HSCT in patients presenting with rapidly progressive scleroderma at risk of organ failure[5].

In RA, phase I/II studies demonstrated feasibility and suggested efficacy in small cohorts, but initial clinical responses were followed by relapse in most patients. With the emergence of biologics, the application of HSCT to RA has largely ceased[6–9]. In 2006, Burt and colleagues reported an uncontrolled trial of HSCT in severe, treatment refractory SLE; 50 patients were enrolled and 50% achieved drug free remission (defined as no immunosuppression except hydroxychloroquine and prednisolone at a dose of below 10mg); there were two treatment-related deaths[10]. A further uncontrolled study has shown similar results in 22 refractory lupus nephritis patients, 82% achieved complete remission after a median of 72 months, five year disease free survival was 53% and one patient died as a result of treatment[11].

Outside the field of rheumatology, MS appears to respond well to HSCT, particularly when used earlier in disease. The data are reviewed comprehensively by Muraro and colleagues[12]. Registry data for European patients with autoimmune disease are maintained by the European Group for Blood and Marrow Transplantation (EBMT). 900 patients were treated with HSCT for autoimmune indications between 1996 and 2007. Figures for progression free survival are encouraging at 55%, 63% and 54% for MS, SSc and SLE respectively, but only 23% in RA.[13].

Summary

HSCT has shown promise in producing long lasting remission of autoimmune disease, albeit with significant treatment-related morbidity and mortality. Consequently, patients enrolled into clinical trials have the most severe and recalcitrant disease phenotypes. Interestingly some trials have suggested that, even where HSCT has not produced drug-free remission, symptoms may become more readily controlled by previously ineffective agents, suggesting immune modulation albeit falling short of tolerance induction[9]. The therapeutically aggressive nature of HSCT means it is not a suitable option for the majority of autoimmune disease sufferers or indeed as prevention.

Lymphocyte targeted monoclonal antibodies

Autoimmunity is mediated by lymphocytes, with CD4 positive T-cells responsible for directing inflammation toward particular auto-antigens (figure 1). Consequently, targeting of lymphocyte subsets is a commonly studied tolerogenic strategy. As with HSCT, lymphocyte modulation confers no antigen specificity and therefore produces generalised immunosuppression, albeit temporary. Lymphocyte modulation by monoclonal antibodies can occur by receptor blockade or cell depletion.

CD3

In the 1980s, following highly encouraging animal data[14], early endeavours in human lymphocyte modulation used mAbs against CD3, a T-cell co-receptor and pan-T-cell marker. The first of these agents, and the first immunomodulatory mAb to be used in humans, was OKT3, a murine mAb initially trialled in steroid-resistant transplant rejection[15–17]**.** Results were strikingly positive, with acute rejection reversed in most cases. Prophylactic trials in renal transplantation were less successful, however, with mixed results also in liver transplantation[18,19]. Furthermore, administration of OKT3 was frequently complicated by a severe first dose reaction, secondary to non-specific T-cell activation and cytokine storm [20]**.**

OKT3 has been superseded by CD3 mAbs with redesigned Fc, to reduce Fc-gamma receptor (FcγR) binding, reducing non-specific T-cell activation and cytokine storms. These include otelixizumab and teplizumab. A small exploratory trial of anti-CD3 treatment in RA has been reported in abstract form[21]: systemic cytokine release accompanied the first dose of therapy but a 'sustained' improvement in symptoms was reported in 3 of 6 recipients. The story in T1D is more advanced, and provides some important messages.

In 2002, Herold and colleagues conducted a Phase I/II study in 12 patients with new onset T1D. A 14 day course of teplizumab produced significant improvements in insulin production at 12 months[22]. In a separate cohort improvement was sustained for at least 24 months[23]. In both cohorts there was a suggestion that a relative CD8+ T-cell lymphocytosis early after therapy predicted benefit. In a controlled trial of 80 patients, a short course of otelixizumab improved c-peptide production and reduced insulin requirement at 18 months[24], and biochemical benefits persisted at 4 years[25]. These studies demonstrate the ability of a single, brief intervention to produce sustained clinical benefit in a chronic autoimmune condition, a defining feature of a tolerance-inducing medicine. A subsequent phase 3 trial of teplizumab, however, studying a range of doses, failed to reach its primary end point[26,27]. Similarly, a study of low dose otelixizumab demonstrated high tolerability but did not replicate the efficacy of higher doses[28].

These findings suggest the potential of anti-CD3 therapy for tolerance induction. From both an immunological and physiological perspective, tolerance is best attempted at the earliest opportunity. Recently, Herold and colleagues administered teplizumab to individuals at high risk for T1D. Participants were non-diabetic relatives of T1D patients, with dysglycaemia and diabetes associated autoantibodies. A 14 day course of teplizumab

reduced progression to established diabetes over the median follow up of two years. One year after treatment only 7% of teplizumab treated participants had developed diabetes compared to 44% of the placebo group[29].

CD4

Several small, uncontrolled studies have investigated CD4 mAbs in patients with treatment refractory RA. Anecdotal reports of positive outcomes alongside a favourable side effect profile were initially encouraging[30]. Larger RCTs, however, failed to replicate the early findings. Van der Lubbe and colleagues conducted a double blind RCT of the anti-CD4 mAb cM-T412 in 60 patients with early, active RA [31]. Treatment led to significant CD4+ T-cell depletion but no benefit was evident after either short or long-term treatment. Similar results were found by others [32,33]. In MS, a phase II trial again failed to meet its primary end point, and cM-T412 was shown to predominantly kill CD45RA+/RO-/Fas- naïve CD4+ T-cells, with a relative preservation of primed CD4+/RO+ cells that are most likely to orchestrate established autoimmunity[34,35].

Whilst disappointing, and less impressive than results with anti-CD3, it is important to consider the bigger picture with CD4 mAbs. Trial designs assumed that anti-CD4 treatment could interrupt active disease after a brief intervention, with suppressed inflammation as the primary outcome. In animal models, however, tolerance develops slowly after CD4 mAb therapy (which does not need to deplete CD4+ T-cells), in part reflecting the induction of immune regulation[14]. In a disease such as RA, there is no reason to expect inflammation to reduce in the interim, unless treatment is also directly anti-inflammatory. Furthermore, the dose of mAb required for tolerance induction is difficult to extrapolate from animal models. Consequently, the initial investigation of tolerogenic therapies should be guided by biomarkers that reflect disease immunopathogenesis rather than clinical endpoints. In this regard, a pilot study combining TNF blockade with a CD4 mAb, designed to be both anti-inflammatory and tolerogenic, was terminated prematurely due to T-cell depletion and lack of short-term efficacy. At long term safety follow-up, however, patients reported improved disease control, exemplified by response to previously ineffective DMARDs, potentially indicating that the primary trial outcomes 'missed' an important benefit of therapy [36].

CD52

CD52 is present on the surface of all mature lymphocytes, providing a target for generalised lymphocyte depletion. CD52 mAbs efficiently harness complement and cellular effector mechanisms to achieve potent and rapid cell death. CD52 mAbs (CAMPATH) have been studied in autoimmunity and transplantation, as well as haematological malignancy. Following renal transplantation, the addition of a rodent IgM CD52 mAb (CAMPATH-1M) to standard immunosuppression reduced acute cellular rejection but increased the risk of serious infection[37]. A number of studies of humanised CAMPATH-1H (alemtuzumab) in RA also provided therapeutic benefit in some recipients, often lasting for 6 months or longer after a brief course of therapy[38,39]. Trials were curtailed largely due to an associated chronic, therapy-induced lymphopenia, plus the emergence of TNF blockade and other targeted therapies. Meanwhile, phase 3 trials in MS showed major reductions in relapse rates, MRI inflammatory lesions, and accumulation of disability following two short courses of treatment 12 months apart[40]. Whilst these outcomes may not reflect robust immune tolerance, they have led to the licensing of alemtuzmab as Lemtrada® for the treatment of MS.

An important adverse, and somewhat paradoxical, effect of alemtuzumab in MS patients is the emergence of secondary autoimmunity. Often this is relatively benign and treatable, such as thyrotoxicosis, but in some cases fatal and life-threatening such as immune thrombocytopenia and Goodpasture's syndrome[41]. Secondary autoimmunity occurs in parallel with reconstitution of the depleted immune system and may reflect homeostatic expansion of autoreactive memory T-cells rather than reconstitution by thymically derived naïve

T-cells[42]. A recent attempt to reduce secondary autoimmunity by thymic stimulation, however, actually resulted in fewer recent thymic emigrants leading to premature study termination[43].

Co-stimulation Blockade

T-cell activation requires a first signal in the form of its cognate antigen and a second co-stimulatory signal to confirm pathological context. Interfering with co-stimulation has therefore been employed as a therapeutic strategy for tolerance induction, with the CD28-CD80/86 interaction of particular interest.

Abatacept, a soluble form of CTLA4 (CTLA4-Ig), interferes with signal 2 by competing with CD28 for binding to CD80 and CD86[44]. It is licenced for the treatment of RA although is administered chronically, in line with other DMARDs. Nonetheless, abatacept's potential to achieve drug free remission has been investigated in several studies, such as AVERT, whereby RA patients with less than 2 years' of symptoms were randomised to methotrexate alone, abatacept alone or combination therapy for 12 months. Those in remission at 12 months had medication withdrawn At 6 months, 17% of patients in the methotrexate group remained in remission as compared to 28% and 25% in the abatacept and combination groups, respectively[45]. The ADJUST trial investigated the ability of abatacept to prevent progression of undifferentiated arthritis to established RA after a 6 month course of therapy. Progression to RA was numerically but not significantly reduced with abatacept (46% versus 67% with placebo) [46]. Interestingly, approximately 10% of patients became anti-CCP negative following 6 months of abatacept, compared to none of the placebo group. They remained negative 6 months later, potentially suggesting immune modulation. In a study that parallels the treatment of patients at high risk of T1D with CD3 mAbs, APIPPRA has randomised patients with 'seropositive arthralgia' to abatacept or placebo, and studied progression to RA[47]. Results are awaited but a delay in progression to RA will support tolerogenic potential of co-stimulation blockade.

Summary

A number of T-cell targeted mAbs have been applied to RA and other autoimmune conditions, in attempts to induce tolerance. Apart from occasional opportunistic infections in association with alemtuzumab, these therapies have proved acceptably safe. Abatacept, in particular, has a long track record of safety in RA. As such, these therapies may have a role in both prevention and treatment of autoimmunity if their tolerogenic potential can be demonstrated. For those agents that deplete T-cells, such as alemtuzumab and some anti-CD4 mAbs, the distinction between tolerance and immune suppression becomes more difficult to prove. Following lymphocyte depletion RA patients appear particularly prone to long-term lymphopenia (but interestingly not secondary autoimmunity), perhaps secondary to defective homeostatic mechanisms[48,49]. Experimental tolerance induction actually appears more robust when induced with non-depleting T-cell mAbs[50]. In this context, abatacept does not deplete T-cells, and lymphopenia with anti-CD3 appears transient.

Antigen Specific tolerance induction

Tolerance via administration of antigen/peptide

Dendritic cells continually encounter, process and present antigens to T-cells. In health, these antigens are of no threat, such as dietary and self-proteins, so dendritic cells suppress, rather than activate, cognate T-cells[51]. This natural mechanism of peripheral tolerance is exploited by researchers developing tolerogenic peptide therapeutics. The underlying premise is that disease-relevant autoantigenic peptides, or modifications thereof, could interrupt or prevent autoimmunity if presented to the immune system in a 'non-threatening' manner, via suppression of pathogenic T-cells (figure 4).

The choice of peptide(s) is a fundamental issue. In some conditions the antigen is clearly defined and of clear pathogenicity, such as the thyrotropin receptor in Grave's disease. In other conditions, such as RA, there is no single autoantigen with a confirmed pathogenic role and multiple autoantigens may be implicated, such as citrullinated proteins[52]. This potential dilemma is often circumvented by invoking the immunological concepts of linked/bystander suppression and infectious tolerance. The underlying mechanisms ensure that, once induced to a specific peptide, tolerance 'spreads' to encompass other relevant peptides and autoantigens (for example derived from the same protein and other proteins in the same tissue)[50]. Extrapolated to the therapeutic situation this implies that tolerance induction to one or a few peptides may be sufficient to ultimately neutralise all pathogenic T-cell clones.

A related approach to circumvent uncertainty around disease-relevant autoantigens has been the use of heat shock proteins (HSP) as surrogate autoantigens. HSPs are expressed at sites of inflammation and therefore in close proximity to autoantigens, and it has been suggested that they can induce bystander suppression[53]. In 2004 Prakken and colleagues administered the HSP derived peptide, dnaJP1, to RA patients. Treatment was well tolerated with a suggestion of treatment-induced deviation of dnaJP1 specific T-cells towards a regulatory phenotype; clinical outcomes, however, were not reported[54]. More recently, an altered peptide ligand (see below) derived from HSP has undergone a phase 1 trial in RA patients, demonstrating safety and tolerability[55,56].

Some trials have focussed on antigens directly implicated in disease pathogenesis. GAD-alum is an altered peptide derived from glutamic acid decarboxylase, a target of autoimmunity in T1D. Despite positive phase II results, a large phase III efficacy trial did not demonstrate benefit in those with established, early onset disease[57,58]; it was also ineffective as prevention in pre-diabetic, GAD antibody positive children and young adults[59]. Similar results were seen with full length insulin[60,61]. In contrast, administration of a proinsulin derived peptide demonstrated safety alongside evidence of clinical and immunological efficacy. Nineteen T1D patients, within 100 days of diagnosis and positive for HLA-DRB1-0401, received subcutaneous treatment at either two or four weekly intervals; eight controls received placebo. Treated patients showed a significantly smaller reduction in C-peptide levels compared to placebo. Furthermore, whilst insulin requirements rose amongst the placebo treated patients, they remained static in the active treatment group. Responders to treatment demonstrated significantly higher IL-10 production by CD4+ T-cells with proinsulin stimulation ex vivo, and increased FoxP3 expression by circulating Tregs in vivo, suggesting favourable immunomodulation toward the antigen[62].

An MS trial illustrates the potential dangers of this approach. In 2000, a myelin basic protein (MBP) derived altered peptide ligand (APL) was administered to MS patients. APLs are designed to bind to disease-relevant class II MHC molecules but impart a partial, negative signal to autoreactive T-cells. The trial was stopped after 8 patients were recruited due to multiple adverse events, including disease exacerbations, hypersensitivity reactions and cluster headaches. In two cases disease exacerbations were attributed to treatment, based on in-vitro responses of peripheral blood and CSF lymphocytes to autoantigenic peptides[63]. In contrast, administration of a cocktail of MBP-derived peptides (ATX-MS-1467) has demonstrated safety in open label phase 1 and 2 trials, alongside potential efficacy. Subcutaneous or intradermal administration of the cocktail, with dose escalation over 4-8 weeks followed by a target dose for 8-16 weeks lead to a significant reduction in MRI lesions; sustained suppression of lesions for 16 weeks off therapy was observed with a longer, intra-dermal dosing regime[64].

Promising data have also been reported on the use of peptide therapy, encompassing two epitopes from the TSH receptor, in Grave's disease. Therapy involved an 8 week dose escalation phase followed by target dose administration for 10 weeks. Seven of 10 subjects showed improvement in thyroid biochemistry alongside reductions in autoantibodies, which persisted for 12 weeks. In 3 patients thyrotoxicosis worsened during this phase 1 study but treatment appeared safe and well tolerated[65]. Peptides representing immunodominant T-

cell epitopes of gluten have also been developed as potential therapy for coeliac disease. Although safety was demonstrated in a phase 1 study, a subsequent phase 2 trial was terminated prematurely due to lack of efficacy[66].

Specific immunotherapy (SIT) has been tested in allergic disease, using both administration of whole antigen and synthetic peptide. Due to its ability to crosslink IgE, and trigger anaphylaxis, whole antigen administration carries some risk. This was highlighted in a meta-analysis of oral peanut immunotherapy which demonstrated an overall increase in the rate of anaphylaxis and adrenaline use[67]. This contrasts with allergic rhinitis which responds well to subcutaneous, sublingual and intratympanic immunotherapy[68]. Immunotherapy with peptides is also being investigated with most clinical data in feline allergy. Although results have been mixed, intradermal and subcutaneous injection of peptides derived from the protein Fel d 1 improved allergic symptoms in some trials, which correlate with modulation of PBMC responses to allergen[69]. Consistent with immunomodulation, long-term improvements have also been reported following desensitisation[70].

A key issue in peptide tolerance regimes relates to dosing. This returns us to the concept of tolerance biomarkers because, as with any tolerogenic therapy, a 'proximal' biomarker of efficacy is ideally required to guide treatment. The studies in Grave's disease and multiple sclerosis both incorporated dose escalation. This approach is also used in allergy, where 'desensitisation' is more established. Animal studies support dose escalation, suggesting it improves tolerance induction and prevents adverse reactions to higher doses of peptide administered without prior dose escalation. Furthermore, this carefully performed work clearly associated tolerance with development of anergic, suppressive, IL-10 secreting T-cells expressing a variety of negative co-stimulatory molecules. One potential caveat is that this work utilised transgenic mice with a single, autoreactive, T-cell receptor, in contrast to an immune system where antigen-specific T-cells are infrequent [71]. Of note, Alhadj Ali and colleagues demonstrated clinical and immunological benefits of treatment with proinsulin peptide but did not employ dose escalation[62]. It is also possible that different peptide dosing regimes, as well as the affinity of administered peptides for MHC and T-cells, induce distinct types of regulatory T-cells[72].

Summary

Notwithstanding the complications seen with APLs, peptide therapies have been well tolerated. Furthermore, they provide the promise of truly antigen-specific therapy without systemic immunosuppression. These characteristics suggest their potential as preventative therapies[73]. A complicating factor could be the need for individualisation of therapy, determined by tissue type and, possibly, demonstration of autoreactivity – administered peptides must bind to recipient MHC and reflect the individual's autoimmune response. In the era of precision medicine this could be viewed advantageously but would require the development of multiple bespoke therapies or, alternatively, peptide 'cocktails'[74]. There are also numerous other ways by which peptide tolerogenicity can be enhanced, such as by delivering peptide covalently linked to red cells or apoptotic cells, incorporation into tolerogenic nanoparticles[75] or by delivery as a component of cellular therapy. This is an area attractive to biotechnology companies, in turn catalysing investment into tolerogenic approaches. Using such delivery vehicles peptide dose becomes less relevant but treatment dose, duration and route of administration still require optimisation. As with other approaches there is a need for robust biomarkers of tolerance induction to guide therapeutic strategy. The association of potentially efficacious peptide therapy in T1D with antigen-specific IL-10 production ex vivo and increased FoxP3 expression in vivo is encouraging and illustrates the types of biomarkers that may be useful in this context[62].

Cellular Therapies

An emerging strategy for tolerance induction is the administration of cellular products with tolerogenic properties (figure 5). These can be either antigen-specific or non-specific. The area has been extensively reviewed recently and therefore will be summarised here[76].

Tolerogenic Dendritic Cells

It is relatively straightforward to differentiate PBMCs into tolerogenic dendritic cells (tolDC) in vitro. Prior to infusion back into the patient they can be loaded with autoantigen, effectively providing another way to administer tolerogenic peptides. Two phase I trials have been performed in RA. Benham and colleagues administered citrullinated peptide loaded tolDCs ('Rheumavax') intra-dermally to 18 HLA typed RA patients[77] whereas Bell and colleagues administered tolDCs loaded with autologous synovial fluid, into an inflamed joint of 9 RA patients[78]. Both studies demonstrated the safety of the approach and reported anecdotal efficacy. The former study also incorporated multiple biomarker readouts, some of which suggested immune deviation towards tolerance. Recently, a nanoparticulate liposome formulation encapsulating a collagen-II (CII) peptide and 1,25 dihydroxycholecalciferol (calcitriol) was administered subcutaneously to patients with RA, to target DCs in vivo. Treatment appeared safe and there was some evidence of CII-specific T-cell modulation[79].

Regulatory T-Cells.

Regulatory T-cells (Tregs) are a potently tolerogenic cell type[76]. They occur at relatively low frequency in peripheral blood but can be purified and expanded ex vivo. The polyclonal product is not antigen-specific but some protocols incorporate antigen-specific expansion. It is also possible to genetically modify the product to express an autoreactive T-cell receptor or a chimeric antigen receptor (CAR), providing a Treg clone. Animal data have been encouraging, with CAR-Tregs specific for myelin antigen able to supress MS in a mouse model[80]. Polyclonal Tregs have been studied in early phase trials in T1D and Crohn's disease. A case report of Treg administration to a patient with SLE has also been reported[81]. 10^8 deuterium labelled Tregs were infused, enabling them to be tracked in vivo. There was a transient increase in Tregs in peripheral blood and, interestingly, the cells localised in cutaneous lupus lesions. This was associated with a reduction in γ-interferon expressing and a rise in IL-17 expressing lesional T-cells, with no clinical change. Antigen specific Tregs are yet to be trialled in the clinic for autoimmunity.

Low dose interleukin-2 (ld-IL2) treatment expands Tregs in-vivo and has been investigated as a potential route to tolerance induction. Regulatory T-cell homeostasis is dysregulated in SLE, due to an acquired IL2 deficiency, and two small trials of ld-IL2 have provided encouraging results; detailed immunophenotyping of treated patients has shown that ld-IL2 is able to normalise the phenotype and increase the numbers of Tregs as well as reduce the relative abundance of T-helper 17 and T-follicular helper cells[82,83]. The TRANSREG trial studied ld-IL2 in 11 different autoimmune diseases, seeking indications suitable for phase II trials. The primary endpoint was the ability to specifically expand Tregs but not proinflammatory effector cells. This was achieved for several conditions, including RA, SLE and ankylosing spondylitis. Clinical outcome was an exploratory endpoint; results were encouraging but will need to be confirmed in larger trials[84].

Potential problems with ld-IL2 therapy include the need for frequent administration and the potential to stimulate cells other than Tregs, including effector T-cells and NK cells. A recombinant fusion protein, comprising a variant form of IL-2 fused to a human Fc, has been designed to avoid some of these limitations. A first-in-human healthy volunteer study confirmed relative specificity for Tregs, with more sustained effects than ld-IL2, as well as less systemic adverse effects[85].

Mesenchymal Stromal Cells

Mesenchymal stromal cells (MSCs) are immature precursor cells with tolerogenic properties[76]. They are hypo-immunogenic and do not express HLA molecules. Therefore, unlike tolDC and Tregs, they can be prepared from an allogeneic donor with the potential for an 'off the shelf' treatment. Furthermore, they are readily

isolated from umbilical cord blood, Wharton's jelly, bone marrow and even adipose tissue. Numerous early phase trials have demonstrated safety and efficacy in SLE, including clinical responses in patients with refractory disease. However, a controlled trial in lupus nephritis (class III or IV) failed to show any additional benefit of MSC treatment over standard of care and was terminated early for futility[86]. MSCs have also undergone early phase trials in RA, as well as other rheumatic diseases, demonstrating safety and hinting at possible efficacy in the small numbers enrolled[87,88]. Larger, double blind RCTs are awaited.

Summary

Cell therapies are expensive and sophisticated to produce but their success for oncology indications has led to significant investment in production facilities and renewed interest in relevant technologies. To gain a foothold for treatment of rheumatic diseases, however, they will need to offer something over and above other treatments, such as reliable and robust tolerance induction. Alternatively, production would need to become simpler and much cheaper. Clearly an off the shelf therapy would be more practical than the need for autologous derivation. Furthermore, a number of questions remain to be addressed, not least the optimal route of delivery. Unlike conventional biologic therapies, administration via the subcutaneous, intradermal or intravenous routes will lead to quite different cell distributions and therefore pharmacodynamics effects. The optimal approach for any of these therapies, of course, would be in vivo induction, as is already being developed for DCs and Tregs.

Concluding remarks

Therapeutic tolerance research has been ongoing for over 30 years and a breakthrough is awaited. Many clinical trials, however, have focussed on established and refractory autoimmunity, where the true tolerogenic potential of these treatments is more difficult to demonstrate; the need to combine with anti-inflammatory or immunosuppressive medication under such circumstances requires further investigation. Nonetheless, promising data have emerged from the application of HSCT in scleroderma, and the prevention of T1D with anti-CD3 therapy. These findings reflect the fact that, in advanced disease with a diverse autoimmune response, drastic measures such as replacement of the immune system may be required; whereas much earlier in the disease process, particularly before clinical disease onset, lymphocyte modulation may suffice. Trials of antigen specific treatment are at an earlier stage but investment in peptide therapeutics, nanotechnology and cellular therapies have produced positive early phase data, sometimes with encouraging biomarker outcomes. Furthermore, some established disease-modifying treatments, such as co-stimulation blockade, may have tolerogenic potential and the results of relevant studies are awaited.

The major impediments to the development of tolerogenic therapies are the lack of biomarkers that indicate immunomodulation, and clarity regarding the optimal antigen for antigen-specific approaches. Regarding the former, tolerance induction aims to induce a long-lasting immune modulation that prevents pathological autoreactivity. Without being able to measure this robustly, the design of tolerance clinical trials is extremely difficult. Conventional efficacy measures, such as improvements in disease activity, will not necessarily improve until tolerance is established, which may take weeks to months. In contrast, a biomarker that signals appropriate immune modification will encourage perseverance with a particular therapy, and facilitate conventional study designs, which examine dose, duration of therapy, and route of administration. Regarding the optimal antigen, the use of peptide cocktails matched to recipient MHC will reduce the chances of administering the 'wrong' antigen.

Combining tolerogenic approaches should also be considered. Combining tolDC therapy with co-stimulation blockade would be a rational combination. Similarly, expanded and reinfused Tregs could synergise with ld-

IL2 to expand endogenous Tregs. Combining anti-inflammatory and tolerogenic approaches is also logical as alluded to earlier in this review[36].

The good news is that tolerance induction, and the search for relevant biomarkers, has now hit 'prime time'. Large consortia such as the Immune Tolerance Network, RA-MAP[89] and RT-CURE have been established to tackle some of the outstanding questions . Future reviews in this series will focus on some of the issues highlighted here: when to intervene with a tolerogenic therapy, identifying the most appropriate autoantigen, and detecting and utilising tolerance biomarkers.

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RT-CURE: https://www.rtcure.com/

Immune Tolerance Network: https://www.immunetolerance.org/

Figures created with biorender.com

Search strategy: Separate searches for each therapeutic agent/technique. Medline database searched via pubmed by searching for therapeutic (including OR generic names. E.g. anti-CD3 OR teplizumab OR otelixizumab OR OKT3) in clinical trials published in English. Result titles then screened for appropriate indications. Clinicaltrials.gov searched using the same terms, including completed clinical trials. Results screened for appropriate indications. Materials also obtained from authors' personal files.

Figures:

Figure 1: Antigen processing to inflammation

Antigen is taken up and processed by dendritic cells. The resulting antigen specific T-cell phenotype is a function of the microenvironment in which the antigen is processed. If danger signals and proinflammatory cytokines are present, such as pathogen associated molecular patterns (PAMPs), the presenting dendritic cell matures and subsequently presents the antigen to CD4+ T-cells with co-stimulation (signal 2), this promotes their differentiation into proinflammatory effector CD4+ T-cells, such as T helper 1 (Th1) ,Th2, Th17 and T follicular helper cells. These cells recruit CD8+ cytotoxic T-cells; release proinflammatory cytokines such as interferon gamma which activate innate effector cells including macrophages and neutrophils and provide help to B-cells leading to production of antibody. All of these pathways contribute to a microenvironment that promotes inflammation. In contrast, if the dendritic cell encounters antigen in the absence of danger signals, it will not provide co-stimulation upon presentation of the antigen to the cognate CD4+ T-cell. Antigen recognition without co-stimulation can lead to T-cell anergy, clonal deletion and induction of a regulatory phenotype, all actions that promote tolerance.

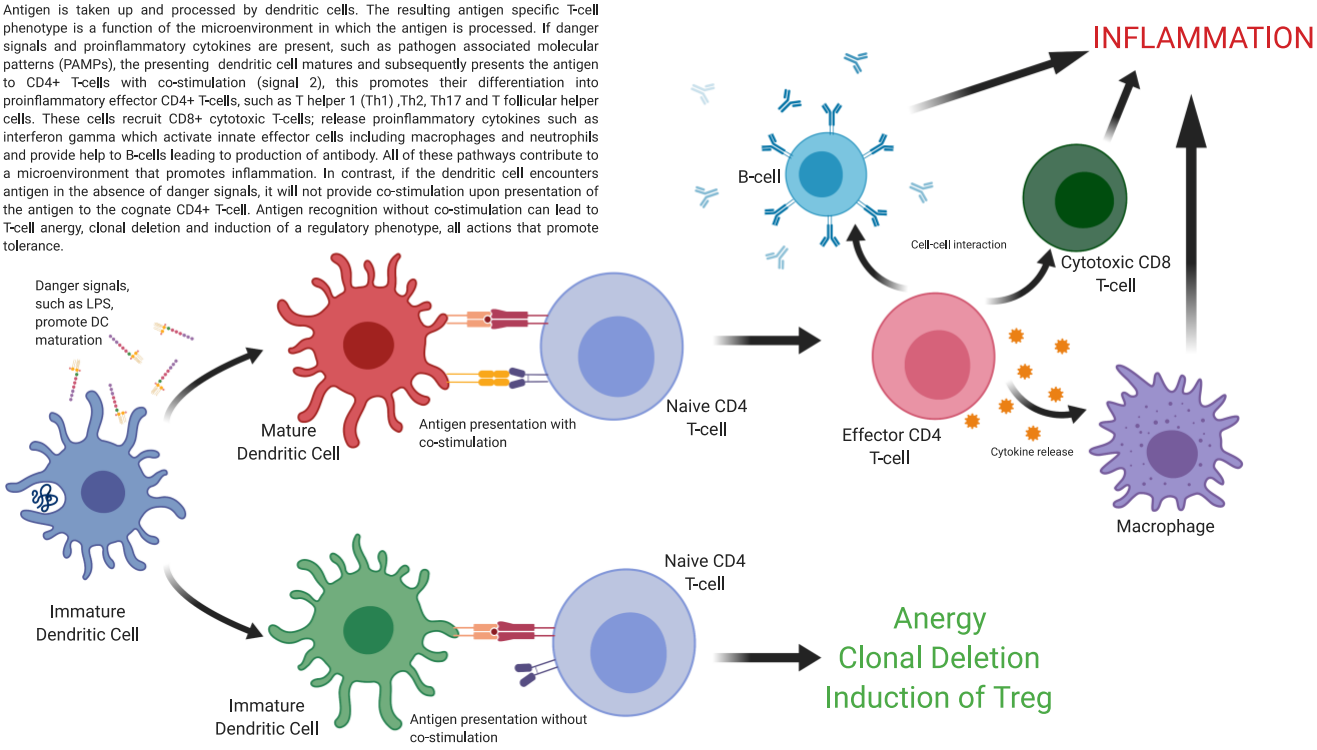


Figure 2: Stages of autoimmune disease

Demonstrated here are the stages of autoimmune disease and how they might most appropriately be treated with different types of tolerance inducing strategy, according to the associated risk. In asymptomatic individuals who are merely at risk of disease, well tolerated, antigen-specific treatments are most desirable. In contrast, in those with established disease and the potential of cure, a higher risk strategy such as lymphocyte depletion may be warranted.

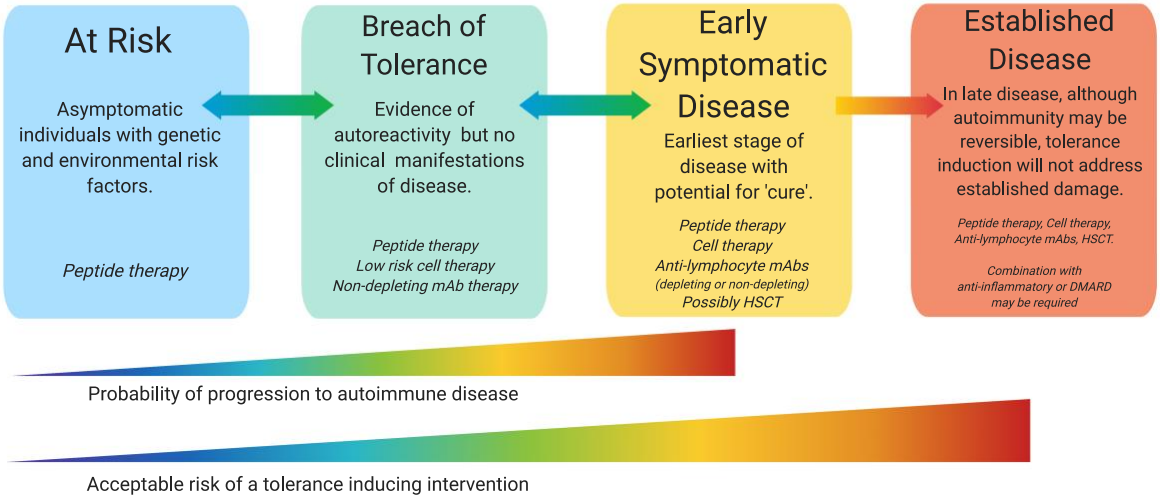
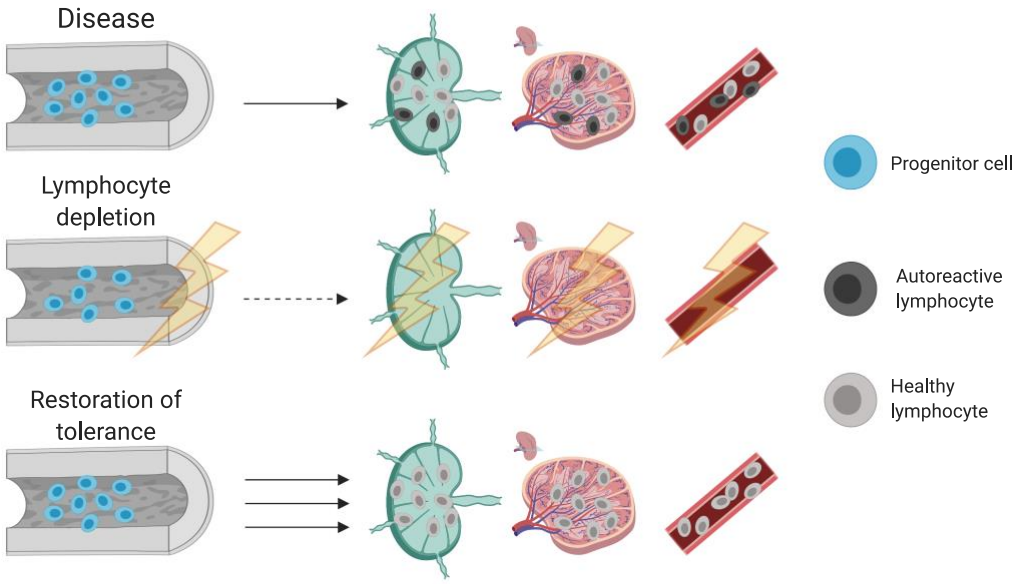
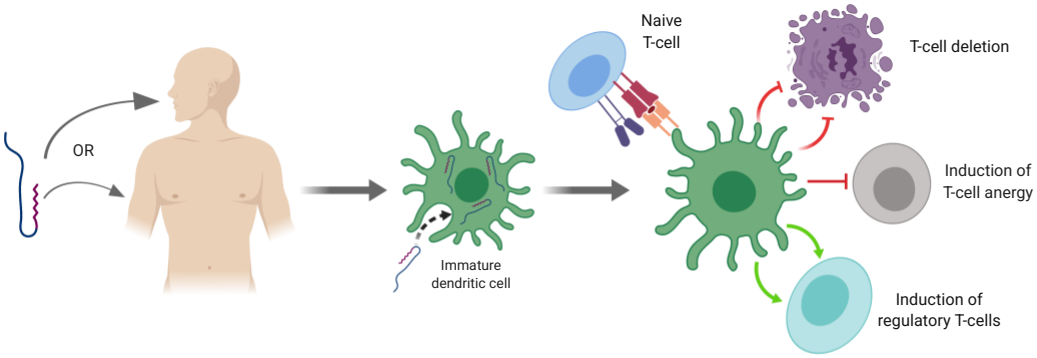


Figure 3: Non-specific lymphocyte depletion



With non-specific lymphocyte depletion, autoreactive as well as healthy lymphocytes are eliminated and replaced from immature progenitor cells. With stem cell transplantation, the new immune system derives from patient (autologous) or matched donor (allogeneic) haematopoietic stem cells obtained from bone marrow or peripheral blood.

Figure 4: Peptide immunotherapy

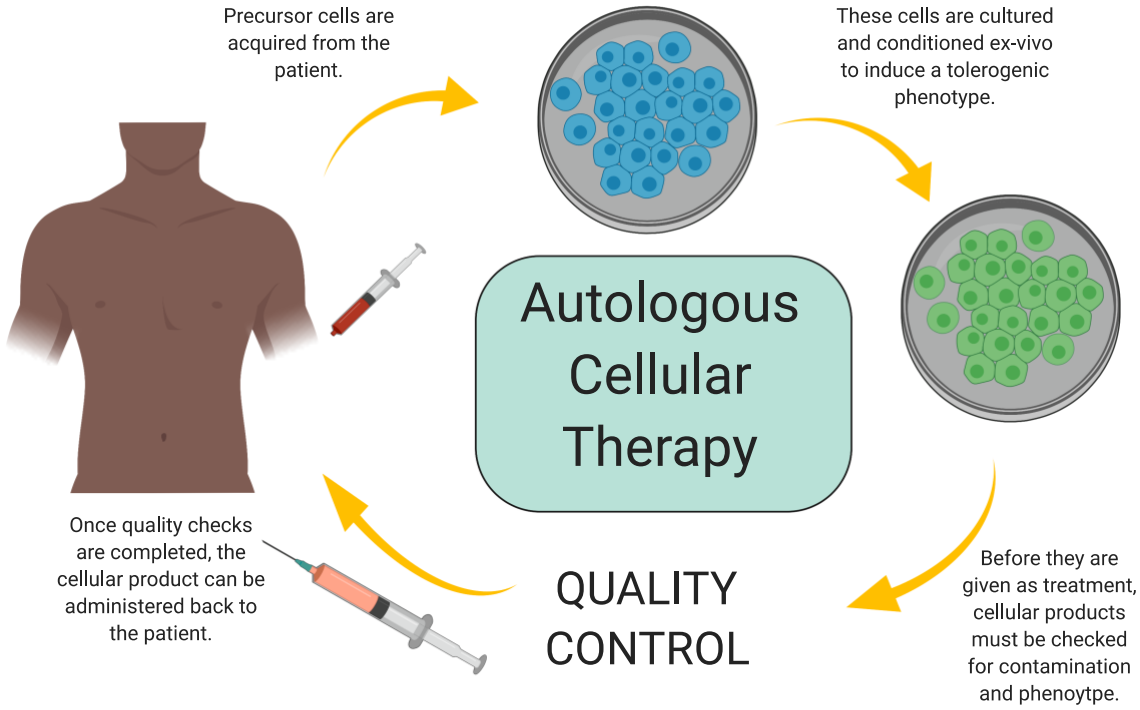


Disease relevant peptides can be administered orally or parenterally.

Following administration, they are ingested by dendritic cells for presentation to T-cells. In the absence of danger signals, the dendritic cells present to T-cells in a tolerance inducing context.

When instructing niave T-cells, immature dendritic cells do not provide co-stimulation (signal 2) to the cognate T-cell, inducing a regulatory phenotype, rendering them anergic or even deleting them.

Figure 5: Autologous cellular therapy



This flow diagram shows the common stages of autologous cellular therapy. The exact methods of cellular acquisition, culture, conditioning and quality testing varies between different cell therapy modalities.

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Autoantigens in rheumatoid arthritis and the potential for antigen-specific tolerising immunotherapy

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Competing interest statement

RT is an inventor of patents supporting technology for antigen-specific immunotherapy, commercialised through UniQuest, UQ's technology transfer company. DCW serves as Chief Scientific Officer for Apitope International NV on a consultative basis. The other authors declare no conflicts of interest.

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Author contributions

HN performed the literature search, and produced the initial draft and figures with RT. RT, VM and DW devised the article, reviewed and edited all drafts and figures. The final draft was approved by all authors.

Summary

Autoimmune diseases, including rheumatoid arthritis (RA), develop and persist due to the failure of immune self-tolerance, which has evolved to regulate inflammatory responses to injury or infection. After diagnosis, patients rarely achieve drug-free remission, and although at-risk individuals can be identified with genotyping, antibody tests and symptoms, RA cannot yet be successfully intercepted. Precision medicine is increasingly offering solutions to diseases that have been incurable. Immunotherapy has begun to achieve this in cancer. However, modulating autoantigen-specific immune responses with immunotherapy for cure of autoimmune diseases is at a relatively immature stage when compared with cancer. Current treatments, using non-specific immune/inflammatory suppression, increase susceptibility to infection and are rarely curative. However, early stage clinical trials suggesting that immunotherapy may achieve prolonged remission and even prevention of progression to diagnosis open new opportunities for tolerance in RA. Here we focus on antigens and antigen-specific tolerising immunotherapy in RA.

Search strategy and selection criteria statement

Referenced peer-reviewed international journal articles or book chapters for this manuscript were identified through searches of PubMed for works that were published from August 1968 to April 2020. Relevant material was identified through the use of search terms that included: “Autoimmune diseases”, “Rheumatoid Arthritis”, “autoantigen”, “autoantibody”, “immunotherapy”, “tolerising”, “epitope”, “citrullinated”. While the use of “citrullinated” may introduce some bias, the large amount of literature and diagnostic antibody test for citrullinated autoantibodies warranted its inclusion. In some cases, reference lists of background articles or studies of high importance were screened to help identify relevant citations. Potential grey literature sources of relevant material, including entries in trial registries, pharmaceutical company/university websites, as well as prominent conference abstracts were also referenced. No language restriction was applied to search criteria. All citations were imported into an electronic database (EndNote X.9) for management.

INTRODUCTION

Immunological tolerance

Immune tolerance towards self-antigens (self-tolerance) for B and T lymphocytes is maintained through central and peripheral tolerance mechanisms. B and T cells differ in the way they "see" antigen, with T cells responding to peptide fragment epitopes of antigen, whereas B cells often recognise conformational epitopes of the whole protein. During an immune response especially to infectious antigen, B cells introduce improvements to the structure, and thereby the antigen-recognition, of their immunoglobulin receptor (BCR) through the process of affinity maturation. Thus, B cells are critical contributors to effective immunity towards the ever-changing landscape of viral and other microbial antigens. The T cell receptor (TCR) on the other hand does not change once formed, but a single TCR has the potential to recognise multiple epitopes with varying affinity (1, 2). Furthermore, CD4 T cells control the function of other arms of the adaptive immune response, including B cells, dendritic cells (DC), CD8 cytotoxic T cells (CTL) and macrophage mediators of innate immune inflammation. For this reason and the fact that most regulatory T cells (Treg) are derived from $CD4^+$ cells, this arm of the immune system bears most responsibility for peripheral self-tolerance. We will, therefore, focus most of our attention on $CD4^+$ T cell tolerising strategies, as the adaptable BCR is a less attractive target for antigen-specific tolerance than the static TCR.

Self-reactive T and B cells are present in blood and lymphoid organs of all people, whether or not they have an autoimmune disease (3, 4). Negative selection of newly-generated autoreactive B cells in bone marrow (central tolerance) is enforced through BCR editing and clonal deletion. Peripheral tolerance mechanisms include anergy (receptor-mediated antigen encounter functionally inactivates the cell), follicular exclusion, apoptosis, BCR revision and immune regulation by Treg (4). After deletion of the most self-reactive T cells during thymic development (central tolerance), further peripheral tolerance mechanisms in lymphoid organs limit the potential of emerging self-reactive T cells to cause damage. These mechanisms include ignorance (antigen is sequestered from the immune system), anergy, apoptosis and immune regulation.

The two major types of Treg include $Foxp3^+$ Treg cells and $Foxp3^-$ Tr1 cells. $Foxp3^+$ Treg are generated in the thymus and the periphery, and control self-reactive T cells by cell contact, cytotoxicity and secretion of immunosuppressive cytokines (5). Tr1 cells differentiate from potentially pathogenic memory T cells (Tmem) after repeated antigen exposure, and control immune responses primarily through interaction with antigen-presenting cells (APC) (6). Secretion of interleukin-10 (IL-10) is a key control mechanism (7, 8). Importantly, the frequency and function of Treg can be manipulated *in vivo*, to reactivate suppressed immune responses in cancer or to suppress autoreactivty in autoimmune diseases (9).

DC are APC that pick up antigens in skin and mucosal sites and present them to T cells in draining lymph nodes and larger lymphoid organs. Antigen context shapes DC control of immune responses to environmental antigens. Microbial antigen with pathogen-associated molecular patterns (PAMPs) in context of infection, vaccine adjuvant or inflammation-associated damage (DAMPs) *activate* the Nuclear Factor-kappa B (NF-κB) pathway in DC to stimulate expansion of antigen-specific CD8 and CD4 T cells (10) (Figure 1). DC process antigens into peptides and present them loaded onto Major Histocompatibility Complex (MHC) molecules. On the other hand, "steady-state" DC taking up and presenting antigens in the absence of adjuvants or immune stimuli or when actively suppressed by drugs or other molecules that *inhibit* NF-κB, induce antigen-specific T cell regulation, which *restores*

immune tolerance (11). Understanding this balance opened the door for antigen-specific tolerising immunotherapies to either leverage resting DC or actively suppress DC in the context of antigen delivery to regulate autoreactive T cells and hence control autoimmune diseases, (12, 13).
The Holy Grails for management of RA are disease prevention or long-lasting drug-free remission. These require a safe, durable and specific intervention to suppress autoimmune responses selectively, whilst leaving the rest of the immune system functionally active for control of infectious and tumour antigens. A Holy Grail is 'much desired but never achieved'; however, we believe that various approaches provide proof-of-concept that this goal is now achievable. Two key questions arise: 1. by which mechanism would the antigen be delivered safely and effectively and 2. which antigen(s) would be delivered for RA? Here we briefly review approaches demonstrating proof-of-concept for antigen-specific tolerising immunotherapy of autoimmune diseases, discuss some general considerations for antigen choice in tolerising protocols and antigen identification for immunotherapy of RA.

When to treat?
Adaptive immune responses are implicated in seropositive RA, but as disease progresses innate immune responses, including cell-mediated and immune complex-, complement- and cytokine-mediated mechanisms, increasingly contribute to disease pathology and clinical activity. Thus, the success of tolerance approaches is likely to be greatest in new-onset RA or at-risk individuals, but reduced in longstanding patients with sub-optimal disease control. Furthermore, application of tolerising strategies in established RA or symptomatic at-risk individuals will almost certainly require concomitant symptom control with synthetic or biologic disease-modifying antirheumatic drugs (DMARDs). Development of tolerising strategies in the at-risk period requires evidence of safety and appropriate immune modulatory activity in established RA, criteria for use e.g. risk biomarkers or risk scoring system, and immune biomarkers that can reproducibly characterise and quantify antigen-specific T cells in at-risk subjects. Several trials have examined the potential for short courses of DMARDs to intercept RA in individuals with unclassified arthritis or clinically-suspect arthralgia. These trials demonstrate delay in symptom onset, and preliminary evidence that risk scoring will be useful for identification of suitable patients. However, application of DMARDs in pre-clinical high-risk individuals does not restore tolerance or prevent the need for future drugs. In contrast, a recent landmark phase 2 clinical trial of a single course of the T cell tolerising immunotherapy anti-CD3 (teplizumab), in high-risk individuals with multiple autoantibodies and impaired glucose tolerance, found that the rate of progression to type 1 diabetes (T1D) was halved at 2 years (14). Remarkably, teplizumab had failed to meet its primary end-point in a phase 3 trial in patients with recent-onset T1D, providing important evidence that application of T cell immunotherapy to patients defined by risk biomarkers in the immediate pre-clinical period may be more effective than after onset of symptoms.

Tolerising platforms for antigen delivery
Antigen-specific therapies for autoimmune disease deliver autoantigen in a regulatory context, without or with a delivery vehicle that reprograms APC by modulating NF-κB, by direct antigen presentation to a naturally tolerogenic site, by targeting steady-state APC or the liver tolerogenic environment or by promoting regulatory T cell (Treg) differentiation (reviewed by (15)). Tolerising approaches in development and clinical trials, as well as challenges to clinical translation were recently summarised (16) and we focus here on approaches taken towards the goal of restoring tolerance in RA.

DC play a critical role in maintaining self-tolerance (17, 18), e.g. targeting steady-state DC *in situ* with antigen coupled to DC-selective antibodies induces tolerance in mice (19). Tolerogenic DCs can be generated *in vitro* from human monocytes or murine bone marrow precursors by including NF-κB inhibitors 1,25 $(OH)_2$ vitamin D3 (calcitriol), BAY11-7082 or rapamycin (11, 20, 21). After pre-clinical mouse proof-of-concept in mBSA or collagen II (CII)-induced arthritis (AIA, CIA) (22), two groups translated tolerogenic-DC or "tol-DC" immunotherapy for treatment of RA (23, 24). These open-label phase 1 clinical trials both demonstrated safety of this approach. Immunomodulatory effects on T cells were reported, using exploratory assays of antigen-specific tolerance (23). Further development and standardization of robust assays of immune tolerance will be essential for progress in future clinical trials. Furthermore, the practical drawbacks of cellular therapy, including cost, product standardisation and production logistics, led to development of approaches that deliver antigen to APC *in situ*. In proof-of-concept studies, liposome formulations encapsulating antigen and various NF-κB inhibitors induced antigen-specific tolerance in mice with AIA (12). In mouse pre-clinical studies, liposomes co-encapsulating calcitriol and antigenic peptide promoted the differentiation of antigen-specific Treg, anergy of memory T cells (Tmem), and suppressed proteoglycan-induced arthritis in an antigen-specific manner (25). Peptide/calcitriol liposomes were preferentially taken up by activated PD-$L1^+$ migratory DC, and regulation was PD-L1-dependent. $CII_{259-273}$/calcitriol liposomes proceeded to a double-blind placebo-controlled single ascending dose phase 1 clinical trial in RA patients carrying the RA-associated MHCII alleles HLA-DRB1*04:01 or *01:01 (26).

Peptide (p)-MHC coated nanoparticles induced Tr1 cells directly from CD4 Tmem, and suppressed disease in several mouse models in an antigen-specific manner, including CIA in arthritic HLA-DR4 transgenic mice (27). Soluble peptides represent an alternative tolerogenic strategy in development for RA. Antigen processing independent antigenic epitopes (apitopes) selectively bind the peptide receptive/empty MHC II at the cell surface of steady-state DC *in vivo* after s.c. administration, as empty MHC II are lost upon DC activation (28, 29). In mouse models, apitopes induced antigen-specific tolerance through induction of anergy and Tr1 cells (30). In an open-label phase 1 clinical trial of intradermal thyrotropin receptor (TSHR) peptides in Graves' disease, 7/10 reduced anti-TSHR autoantibodies (31).

Oral antigen tolerised rodent models of CIA through induction of Treg, but required repeated administration of antigen to reach and maintain efficacy (32). A placebo-controlled trial of oral bovine CII in the 1990s in patients with RA suggested better efficacy with lower doses (33), recapitulating mouse pre-clinical studies with a similar dose outcome (34). Today, better understanding of mucosal immunology (35), and improved clinical trial design and bioassays of immune modulation could be applied to advance oral tolerance platforms. Clinical trials of oral heat shock proteins (HSP) dnaJP1 15-mer peptide and i.v. BIP identified potential anti-inflammatory mechanisms (36, 37). In a double-blind placebo-controlled phase 2 trial of RA patients taking hydroxychloroquine or sulfasalazine, oral dnaJP1 was safe. A significant improvement in ACR20 response was observed in patients receiving dnaJP1 relative to placebo, in post-hoc analysis. T cells secreting TNF in response to dnaJP1 significantly decreased after 6 months' treatment. In a double-blind placebo-controlled phase 1/ 2 trial, i.v. BiP was safe, with significant reductions in serum VEGF and IL-8 in BiP-treated RA patients (36, 37).

CONSIDERATIONS FOR ANTIGEN-SPECIFIC IMMUNOTHERAPY

Antigens: general considerations for tolerance

Development of antigen-specific immunotherapy requires an understanding of which antigen/s to target in an autoimmune disease and a decision about antigen format. The choice of antigen format depends to some extent on the product-specific mechanism of tolerance. Antigen formats include single or multiple epitopes restricted by specific disease-associated MHC II molecules, pMHC complexes, whole proteins or protein fragments, RNA or DNA. Both the route of injection and dose influence antigen immunogenicity. For example, s.c. administration of soluble peptides in very low doses is non-immunogenic, while administration of high doses, aggregates or protein promotes immunogenicity through immune complex formation and macrophage/DC activation, leading to induction of CTL and autoantibodies. It may be feasible to administer intact antigen either in a less immunogenic plasmid or particulate formulation that ensures uptake and antigen release within APC. For example, protein antigen coupled to microparticles is taken up and processed by marginal zone macrophages in spleen and liver through the MARCO scavenger receptor (38).

Tissue DC picking up protein antigens locally process and present MHC II-restricted peptides to CD4 T cells and cross-present MHC I-restricted peptides to CD8 T cells in draining lymph node (dLN) and the tissue. To avoid immunogenicity or cytokine storm, most products in development employ a single peptide or a few related epitopes. This is because antigen-specific Treg induced towards a single epitope suppress the function of tissue-draining DC presenting the same epitope, propagating tolerance towards all tissue-specific epitopes those DC present. This process, known as bystander tolerance (Figure 2), is analogous to the process of epitope spreading, where autoimmunity matures due to presentation of multiple tissue-derived antigens with T cell help from a limited number of antigen-specific T cells in the context of DAMPs (39). Bystander mechanisms of dLN modulation include IL-10 and TGF-β induced upon TCR signalling after antigen engagement, coinhibitory cell surface interactions, and cytotoxicity. Bystander tolerance has been clearly exemplified by tolerogenic protocols in pre-clinical mouse models, modulating immune responses in organs distant to the site of antigen delivery through local autoantigen presentation (27, 40). Tolerising immunotherapy with liposomes encapsulating a single islet CD4 epitope suppressed the progression of autoimmune diabetes in mice after onset of hyperglycemia through bystander suppression of islet-reactive CTL, controlling diabetes progression (41). The advantages of bystander tolerance over cytokine blockers are tissue-restricted immune suppression and longevity, since this mechanism leverages ongoing tissue self-antigen presentation to reinforce Treg activation and memory. Research in pre-clinical models and clinical trials is required to address the impact of bystander suppression on viral or tumor-specific CTL activity. Furthermore, conclusive demonstration of bystander tolerance is needed in clinical trials of antigen-specific tolerance.

Multiple antigens have been described in autoimmune diseases. Human Leukocyte Antigen (HLA) restriction of epitopes poses a potential challenge for immunotherapy, as epitope-specific immunotherapy must be matched to patient HLA-type. Solutions include: 1. Delivering a single strong T cell epitope in a tolerogenic format to patients of appropriate HLA type(s), with perpetuation of regulation through bystander tolerance; or 2. delivering a long antigenic sequence, or cocktail of epitopes covering a range of HLA restrictions to the diseased population, aiming to cover HLA diversity. While each is a valid long-term strategy, the immediate translational challenge of antigen-specific tolerising immunotherapy is to demonstrate consistent antigen-specific immunomodulation

using robust assays in small early-phase trials. While safety, clinical data and non-antigen-specific immunological outcomes could be evaluated from small trials in which multiple epitopes were delivered (23, 42, 43), modulation of antigen-specific T cells to tolerising therapy may be most easily interpreted in a small trial when a single epitope is delivered to patients with a specific or limited number of HLA types restricting that epitope (44). Although tolerising immunotherapy with multiple autoantigens prevented disease in mouse models of MS and T1D, the capacity of a platform to induce bystander tolerance may dictate whether multiple autoantigens are similarly needed to suppress epitope spreading during chronic autoimmune disease in humans.

Identification of autoantigenic epitopes suitable for antigen-specific immunotherapy

Selection of suitable antigenic epitopes for tolerising immunotherapy in autoimmune disease is challenging for several reasons. Firstly, the number of antigenic targets differs greatly between diseases. In antibody-mediated organ-specific autoimmune diseases (such as myasthenia gravis or Graves' disease) and environmentally-driven T-cell mediated disorders (such as coeliac disease), immunogenic epitopes are generally restricted to antigenic hotspots. In systemic autoimmune diseases (such as RA), there are multiple autoantibody specificities, which vary between individuals (Table 1). Secondly, the HLA restriction and binding affinity of antigenic epitopes differs. For peptide-based immunotherapy, HLA restriction within the diseased population must be taken into account. Thirdly, pMHC-specific autoreactive T cell precursor frequencies and TCR affinities differ, and high TCR affinity is important for the induction of Treg (45). Fourthly, autoantigen distribution impacts tissue-specific autoreactive T cell identification and characterisation.

Specific HLA-DR gene variants are highly associated with ACPA+ RA (46-48). Polymorphisms at amino acid positions 13, 71 and 74 of the DRβ chain, encoding the "shared susceptibility epitope" or SE in the HLA-DR antigen-binding groove - found in multiple $ACPA^{+}$ RA-associated DR alleles - confer the highest risk (49). These amino acids contribute to the positively charged fourth anchoring pocket (P4), which preferentially binds uncharged or negatively-charged amino acids, such as citrulline, or aspartic acid in $CII_{261\text{-}273}$ and $CII_{1237\text{-}1249}$Cit1240 (50-52). Thus, potential citrullinated epitopes are not limited to P4Cit (52-54).

To date, HLA-DR binding epitopes predicted in silico from candidate autoantigens have been tested in T cell reactivity assays e.g. proliferation, cytokine production. High levels of background reactivity to autologous APC and T cell anergy to self-peptides limit signal from such assays in RA. However, understanding of self-specific T cells has expanded with the advent of self-peptide-HLA-DR tetramers, to which their TCR bind. In alternative strategies, immunogenic sequences have been identified after autoantigen immunisation of HLA-DR transgenic mice – notably CII (55). Elution studies also characterised canonical sequences binding to specific HLA-SE molecules (51).

In T1D, multiple T cell islet autoantigenic epitopes and their HLA-restriction have been characterised by screening T cell clones grown from islets collected from deceased patients. The islet specificity of T1D restricts antigen presentation to small amounts derived from islet beta cells, which are depleted by the autoimmune response, reducing Tmem reactivation with time. By contrast in RA, cartilage and synovial-derived antigens e.g. CII, fibrinogen, aggrecan or vimentin, are widely distributed in tissues subjected to mechanical stress and damage. Therefore, autoreactive T cells would be frequently and chronically exposed to synovial antigen presentation. In mouse models of transgenic autoantigen, constitutive self-tolerance to widely-expressed antigen was maintained predominantly by deletion or anergy, while antigen-specific Treg maintained self-tolerance to organ-specific transgenic antigen.

Tissue antigen distribution has important implications for future maintenance of antigen-specific tolerance after tolerising immunotherapy in diseases such as RA and T1D. With ongoing presentation of widespread cartilage or synovial-derived antigen and autoantigen-specific T cell anergy in RA, bystander tolerance may be leveraged to maintain tolerance, provided a strong joint-specific antigenic epitope or set of epitopes induce regulatory populations to start the local bystander cascade. Joint inflammation or flare is an early warning of disease re-ignition, and presumably DAMP-associated antigen presentation in RA. In organ-specific diseases with little ongoing antigen presentation accompanied by DAMPs, repeated or sustained antigen delivery may be needed to maintain Treg cell function. Good biomarkers identifying when to dose will be essential to success in diseases lacking overt symptoms, such as T1D.

TCR reactivity, affinity and antigen signalling

HLA binding does not itself identify a relevant T cell epitope, and strength of peptide binding to HLA does not directly predict strength of the cognate TCR interaction. Using pMHC tetramers, Tmem recognising self-epitopes have been characterised in the circulation of RA patients and healthy controls (51, 56, 57). Citrullinated α-enolase-specific Tmem were enriched in SF of HLA-DRB1*0401^{+} RA patients (57).

Multi-colour labeling of individual tetramers demonstrates separate, rather than cross-reactive populations of T cells recognizing citrullinated self-antigens, suggesting multiple potentially autoreactive clones (58, 59). Recent data confirm unique TCR repertoires of T cells of different specificities sorted from PB (58), and the infiltration of ST by CD4 Tmem, including peripheral helper T cells (Tph) and activated CTL (60).

The development of suppressive antigen-specific Foxp3^{+} Treg is an active process resulting from TCR signalling (e.g. by antigen or anti-CD3) leading to calcium flux and NF-kB activation, along with tolerogenic signals derived from APC or the environment such as TGF-β, retinoic acid, and sufficient IL-2 (61). Thus for Treg induction, it is important to target NF-κB inhibitors to APC, rather than systemically. Ongoing exposure to tissue antigen (e.g. in skin) and IL-7 promotes a proliferative, suppressive, memory Treg phenotype (62). Similarly, IL-10^{+}Foxp3^{-} Tr1 cells develop from antigen-specific Tmem in response to chronic antigen stimulation in the presence of tolerogenic signals, such as IL-27 or IL-10 (63).

Are there key autoantigens in RA?

In RA, multiple T cell and B cell epitopes characterise the autoimmune response. Anti-citrullinated peptide antibody (ACPA) autoimmunity is observed in 70% of RA patients. In longitudinal studies of ACPA in at-risk subjects developing RA, autoreactivity usually started to one citrullinated epitope at sero-conversion, but without preference for any particular peptide. This is not surprising, as anti-citrullinated peptide antibody (ACPA) responses are highly cross-reactive toward linear citrullinated epitopes (64, 65). Indeed, crystal structures of ACPA in complex with citrullinated epitopes demonstrate that recognition of citrulline and the neighbouring peptide backbone permits extensive cross-reactivity at peptide and protein levels (65-68). Further, smoking associates with development of low titre ACPA and other autoantibodies, rather than inducing autoimmunity towards a particular citrullinated epitope specificity (69). These data strongly suggest that in pre-clinical RA, ACPA autoimmunity does not initiate towards a single specific citrullinated peptide and that ACPA linear epitope reactivities do not identify an initiating antigen.

Ge and Holmdahl provided a conceptual framework based on evidence from mice. They suggest that ACPA-producing B cells escaping negative selection survive and expand only with T cell help. These T cells are neither likely to represent a single antigen specificity, nor are necessarily citrullinated epitope-specific (70). Indeed, RA candidate autoantigenic T cell epitopes include a growing number of citrullinated and non-citrullinated peptides restricted by RA-associated HLA class II molecules, none of which is dominant amongst RA patients (Appendix 1), and several potential CTL epitopes (71). This is not necessarily a problem for designing tolerising antigen-specific therapy that invokes bystander tolerance by driving antigen-specific Tmem to Tr1 cells: any strong joint-specific T cell epitope in tolerogenic format will likely promote antigen-specific bystander regulation (27). However, it remains to be determined whether bystander regulation can be improved with multiple epitopes, potentially targeting different diseased tissues or autoantibody specificities. Assays that simultaneously and reproducibly measure immune responses to multiple antigenic epitopes will be useful to answer this question.

How then might citrullinated autoantigens contribute to RA?

Citrullination is catalyzed by a family of peptidyl arginine deiminase (PAD) enzymes. While stored in cytoplasmic granules, in the nucleus PAD4 citrullinates histones in chromatin of DNA. In neutrophils, PAD4 is activated by various inflammatory signals and inactivated by apoptosis. Microbial activation may induce neutrophil death associated with the formation of extra-cellular traps (NETs). PAD4-dependent extruded nuclear material includes chromatin histones entrapping microbes and their products (72). RA patients' PB and SF neutrophils were found to citrullinate multiple proteins and form NETs more readily than healthy control PB neutrophils. This "hypercitrullination" is also PAD2 or PAD4-dependent and requires perforin/granzyme B or complement-mediated cell lysis. If phagocytized by DC, neutrophil NETs may contribute multiple citrullinated autoantigens, including PAD4 itself and fibrinogen, vimentin, α-enolase, histone H1-4 for T cell activation (73).

While mechanistic studies have yet to demonstrate the causality of particular microbes in RA development, a plausible hypothesis is that autoreactivity is primed at mucosal sites, associated with chronic microbial dysbiosis in people at genetic and environmental risk (74). Microbial factors, NK cells or CTL may drive neutrophil hypercitrullination and presentation of shared antigens in mucosal sites and synovium, driving restimulation of antigen-specific Tmem at different sites. Indeed, a comparison of citrullinated epitopes in lung and ST of RA patients identified two citrullinated vimentin epitopes shared between tissue sites (75). One of these was included in a trial of DC immunotherapy, and antigen-specific suppression of cytokine production to it was shown (23).

Development of ACPA is unusual in animal models of inflammatory arthritis (76-78) and no reproducible model of citrullinated epitope specific autoimmunity has been developed in animals, thus limiting studies of citrullinated peptide-specific tolerance. Immunization of DR4 transgenic mice with citrullinated-vimentin$_{59\text{-}79}$ induced antigen-specific T cells but not disease (79). On the other hand, LPS-treated lymphocyte-deficient mice developed bone loss after transfer of ACPA (80). Furthermore, while H-2^{q} mice immunised with citrullinated histone-H2B developed ACPA, arthritis developed with concomitant low-dose bovine CII immunisation, implicating H-2^{q}-restricted CII-specific T cell help in disease amplification. ACPA and arthritis were not induced by low-dose CII immunisation alone (81). These studies demonstrate in mice that generation of ACPA does not necessarily correlate with development of arthritis (as in human), but that ACPA may exacerbate local inflammation, arthritis or osteoclastogenesis.

METHODS FOR ANTIGEN DISCOVERY

Mass spectrometry

Various proteomic approaches have been used to identify synovial autoantigens in RA, including immuno-affinity purification of HLA-DR molecules from synovial tissue (ST), synovial fluid (SF) or PB samples, followed by mass spectrometry (LC-MS/MS) identification of eluted peptides (82). Using this approach, Wang and colleagues identified HLA-DR-restricted self-peptides, of which 2 – from N-acetylglucosamine-6-sulfatase (GNS) and filamin A – were highly expressed and immunogenic in HLA-DR SE+ RA patients. Recent advances in MS instruments and methodologies have also enhanced discovery of citrullinated autoantigenic targets in RA (83). While citrullination was enriched in inflamed joints of RA patients, and >100 identified, citrullinated candidate autoantigens were expressed in tissues from both healthy controls and RA patients (84), highlighting that antigen citrullination is not exclusive to RA.

Screening T cells to identify relevant antigens, and preclinical assays in RA

Biomarkers that quantify and phenotypically characterise the antigen-specific immune response are critical components for any clinical trial of antigen-specific tolerance. pMHC-tetramers quantify rare antigen-specific CD4 and CD8 T cells, and flow cytometry and cell sorting can be used to characterise their phenotype and function. For example, in arthritic humanised DR4 and DR1 mouse models of arthritis, $CII_{260\text{-}272}$-specific T cells were shown using tetramers to expand in lymph nodes draining antigen priming, and to express high levels of T helper (Th)1 and Th17 cytokine mRNA. These T cells exhibited a high degree of clonality, and infiltrated ST soon after the onset of inflammation (55, 85). This sequence of events provides a hypothetical model for analysis of oligoclonally-expanded T cell populations in LN or ST of patients with recent-onset RA.

Assays using pMHC tetramers, with and without antigen-specific proliferation assays and intracellular cytokine staining, allow researchers to follow antigen-specific T cells in mouse models of disease, and in RA clinical studies (25, 51, 59, 71, 86). pMHC tetramers are also useful reagents to document TCR usage in single antigen-specific T cells. pMHC tetramers can be used in combination with T cell response assays in vitro to identify disease specific peptides of relevance and their HLA restriction. Clonally-expanded TCRα and TCRβ pairs can be cloned into TCR deficient cell lines and screened for pMHC binding and peptide reactivity. Furthermore, high-throughput single cell RNA sequencing using barcoding technology links the whole transcriptome, including TCRα and TCRβ, in one parallel assay from complex samples, such as RA ST. Further research is needed to characterize clonotypically expanded ST CD4 and CD8 TCR and their antigenic targets, to provide confidence that autoantigens employed in tolerogenic protocols are relevant to Tmem in RA joints.

RA autoantigen candidates

Many RA candidate autoantigens are described, including native and post-translationally modified (PTM) proteins, and a growing number of T cell epitopes. In Appendix 1 and below we briefly summarise features of some of the major antigens studied in pre-clinical models and, in some cases, clinical trials.

Aggrecan is a proteoglycan that forms part of the major structural component of cartilage. The aggrecan G1/G2 region appears to be an antigenic hotspot, with multiple citrullinated and non-

citrullinated epitopes restricted by several MHC class II molecules in human and mouse respectively (87-89).

CII is an articular cartilage autoantigen, for which there is extensive evidence of immunogenicity in multiple animal species, CII-autoreactive CD4 T cells and B cells and dominant T and B cell epitopes overlapping between species (90, 91). CII-specific autoantibodies are directly pathogenic upon transfer to mice (the CAIA model) (92), through direct binding of the autoantibodies to articular cartilage and complement fixation, with a local inflammatory response that models the effector phase of autoimmune arthritis.

Human cartilage glycoprotein 39 (HC gp-39) is secreted by articular chondrocytes and synovial cells. Serum levels correlate with joint inflammation in RA patients and an inflammatory mouse model of glucose-6-phosphate isomerase (GPI) induced arthritis. HC gp-39 was recognised by antigen-specific Treg. HC gp-39 peptide-specific responses have been identified in HLA-DRB1*0401 RA patients and may induce suppressive responses by Treg (93, 94).

Multiple NETs-associated citrullinated peptides have been shown to bind ACPA. $H4_{33\text{-}47}$Cit39 bound ACPA from the majority of RA patients and Indigenous North American first-degree relatives, suggesting H4 might be an antigenic hot spot in the developing RA autoimmune response (95). Calreticulin is an interesting autoantigen identified by screening a synovial B cell-derived monoclonal antibody, for binding to fibroblast-like synoviocytes (FLS) but not NETs (96).

Heat shock proteins (HSPs) are intracellular chaperones, whose expression is upregulated during joint inflammation, necrosis or heat stress. HSPs are immunogenic and can also induce Treg. HSP70 is anti-inflammatory when released from RA FLS, through Treg immunomodulation. The sequence of HSP dnaJp1 is homologous with the HLA-DR SE sequence.

Heterogeneous nuclear ribonucleoprotein (hnRNP)-A2 (RA33), is the target of anti-RA33 autoantibodies, toward which both B and T cell epitopes are described. Recombinant hnRNP-A2 stimulated strong proliferative and cytokine responses in T cells from RA and lupus patients (97). Patients with anti-RA33 may be ACPA and RF seronegative.

B CELLS

B cells should also be considered as APC targets for immunotherapy in seropositive autoimmune diseases, such as RA, due to selective antigen uptake via their B cell receptor (BCR). They play a key role in disease perpetuation after onset. The attraction of targeting and killing antigen-specific B cells in RA is that they only represent a small proportion of total B cells. This more specific approach may be less toxic than B cell depletion agents such as anti-CD20. However, the challenges are that many antigen-specific memory B cells and plasmablasts may reside in the tissues rather than in the circulation, and IgG antibody-producing plasma cells no longer express antigen-specific BCR. Although many B cell epitopes are conformational, B cell immunity to citrullinated proteins is primarily driven by short motifs, so the RA disease setting may be especially well-suited to test such an approach. As a proof-of-concept, antigen-specific B cell tolerance was achieved in RA memory B cells in vitro and in mice with liposomes co-conjugated with a cyclic citrullinated peptide and a CD22 ligand (98).

CONCLUSIONS

Figure 3 presents a summary model of the progression of autoimmunity and the interplay with innate immunity in the period leading up to clinical expression of RA. As we discuss here, many widely-

expressed autoantigens participate in RA autoimmunity, including those derived from NETosis, cartilage, and other cellular components, and these are likely cross/presented to CD4 and CD8 T cells at various stages of disease development. Despite the specificity of ACPA for seropositive RA, the inherent cross-reactivity of ACPA towards linear epitopes and the broad spectrum of observed T cell reactivities reinforce that there is no single dominant autoantigen. On the other hand, this opens opportunities for restoring tolerance before and at onset of RA, with antigen-specific immunotherapy using one or more of the described epitopes to drive bystander suppression, combined with conventional or biologic DMARDs to control inflammation driven by innate mechanisms. A choice of platforms exists for antigen-specific immunotherapy, some of which have been tested in the clinic, including apitopes, peptide/immunomodulator nanoparticles, cellular therapies, and direct B cell targeting. Combination approaches may be particularly suited for long-term control or prevention of RA and of other seropositive autoimmune diseases, including systemic vasculitides and systemic lupus erythematosus.

Table 1: Autoimmune diseases and their auto antigenic targets

Autoimmune disease	Autoantibodies	Identified or potential autoantigens
Autoimmune Addison's disease (AAD)	Anti-21-OH, anti-17-OH, and anti-SCC	
Autoimmune hepatitis (type I and II)	ANAs, ASMAs, AAAs, anti-SLA/LP, AMAs, ALKM1, ALKM-3	ASGPR, CYP2D6, SLA/LP
Autoimmune renal vasculitis:		
Goodpasture's syndrome	anti-glomerular basement membrane	ANA, GBM, Collagen IV α3 135-145
MPO-ANCA vasculitis	MPO-ANCA	MPO
PR3-ANCA vasculitis	PR3-ANCA	PR3
Autoimmune thyroiditis:		
Graves's disease		TSHR
Hashimoto's thyroiditis		TPO
Idiopathic inflammatory myopathy:		
Polymyositis	Anti-Jo-1, anti-Mi-2, anti-SRP,	Histidyl tRNA synthetase, aminoacyl tRNA synthetases, signal recognition particle
Dermatomyositis	anti-TIF1γ/α	Transcription intermediary factor 1γ/α
Multiple Sclerosis	Oligoclonal bands, anti-MOG, anti-MBP	α-enolase, β-arrestin, MBP, MOG, PLP, S100B, RASGRP2
Neuromyelitis optica	Anti-acquaporin-4	Aquaporin-4
Primary biliary cholangitis	Antimitochondrial antibodies (AMAs)	Pyruvate dehydrogenase complex E2 subunit (PDC-E2)
Rheumatoid arthritis	RF, anti-PAD3/4, anti-CarP, anti-RA33, anti-collagen II	Collagen II, HC-gp 39, hnRNPA2, IgG, HSP,
	ACPA	Citrullinated: H3, H4, α-enolase, vimentin, collagen II, aggrecan, PAD4, CILP.
Sjogren's syndrome	Anti-SSA, anti-SSB, RF, ANA, anti-M3	Ro, La, M3
Systemic sclerosis	Anti-topoisomerase I, anti-U3 RNP, anti-centromere	DNA topoisomerase I, RNA polymerase III, fibrillarin, Th/To RNP, centromere proteins
Systemic lupus erythematosus	Anti-nuclear (ANAs), anti-dsDNA, anti-Sm	dsDNA, nucleosomal histones, snRNP, poly(ADP-ribose) polymerase, Sm antigens of U-1 RNP

	anti-phospholipid	Phospholipid-β-2 glycoprotein I,
Type I Diabetes	GADA, anti-insulin, anti-ZnT8A, anti-IA2A	Chromogranin A, IA2A, IGRP, proinsulin

AMAs = Antimitochondrial antibodies; GPI = glucose-6-phosphate isomerase; PAD = peptidyl arginine deiminase; CarP = carbamylated proteins; hnRNPA2 = Heterogeneous nuclear ribonucleoproteins A2; ANA = Anti-nuclear antibodies; anti-Sm = anti-Smith; snRNP = small nuclear ribonucleoproteins; GADAs = Glutamic acid decarboxylase antibodies; IA2As = insulinoma-associated protein 2 antibodies; ZnT8As = Zinc transporter 8 autoantibody; MOG = Myelin oligodendrocyte glycoprotein; MBP = myelin basic protein; ASMAs = Anti-smooth muscle antibodies; AAAs = Antiactin antibodies; SLA/LP = soluble liver antigen/liver pancreas; ASGPR = Asialoglycoprotein receptor; CYP2D6 = Cytochrome P450 2D6; ANCA = anti-neutrophil cytoplasmic antibodies; ALKM1/3 = Anti-liver-kidney microsomal-1/3 antibodies; SSA/B = Sjögren's-syndrome-related antigen A/B; M3 = muscarinic acetylcholine receptor; TPO = thyroid peroxidase; Tg = antithyroglobulin; TSHR = antithyrotropin receptor; OH = hydroxylase; GBM = glomerular basement membrane: CILP = Cartilage intermediate-layer protein.

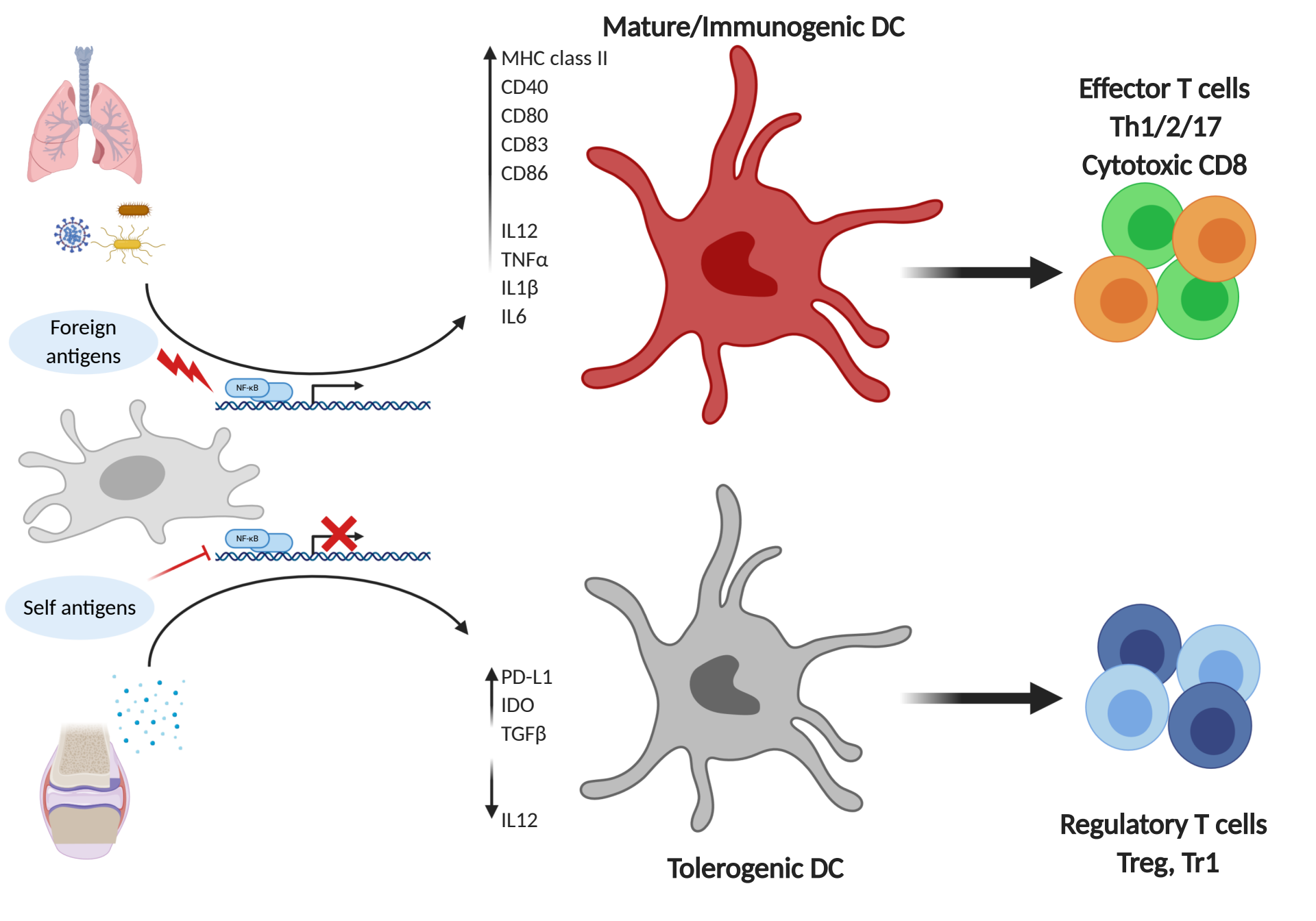
Figure legends

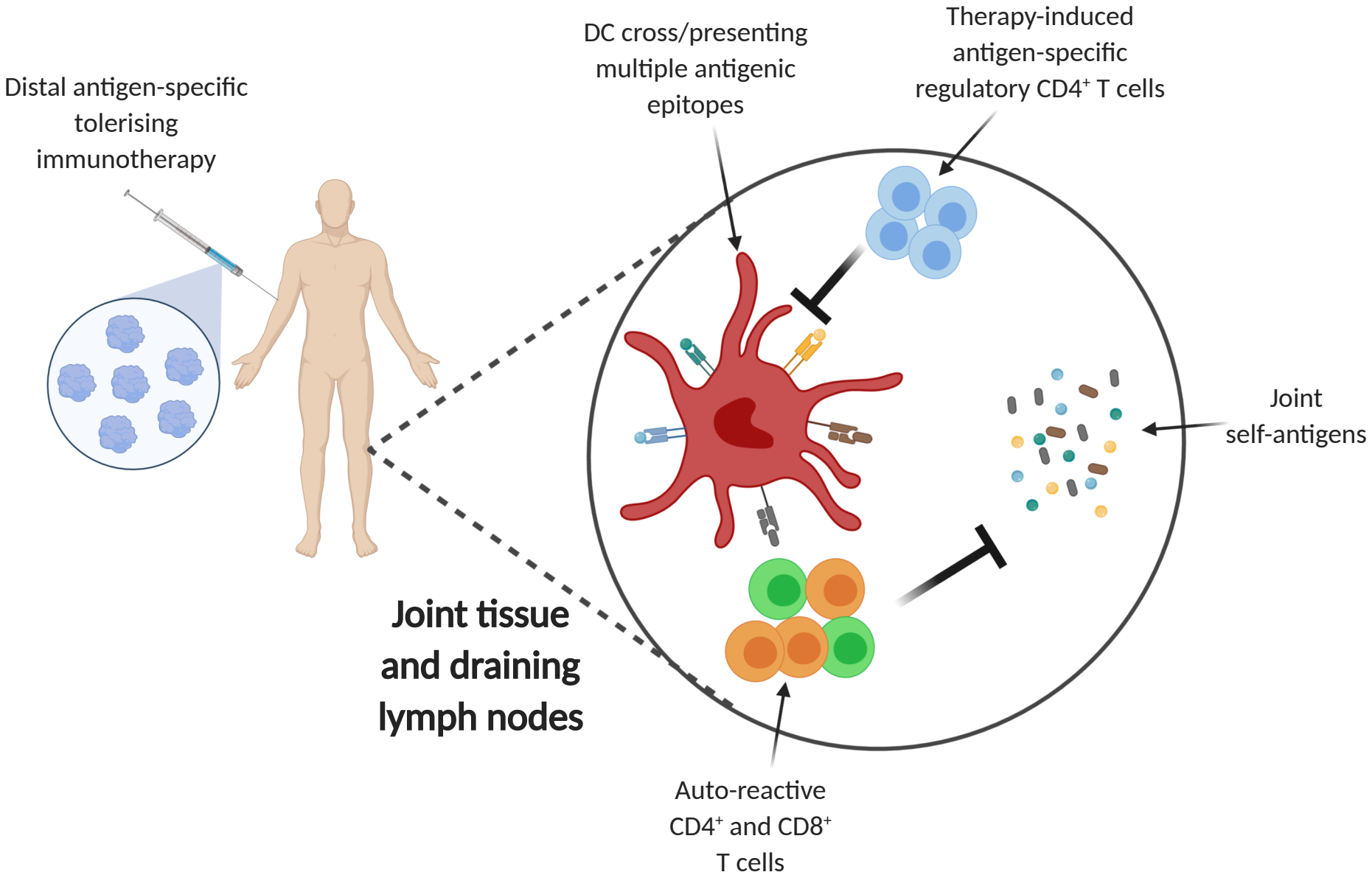
Figure 1. Contextual activation of DC determines T cell fate. The environmental context as DC pick up antigen promotes DC activation or tolerance.

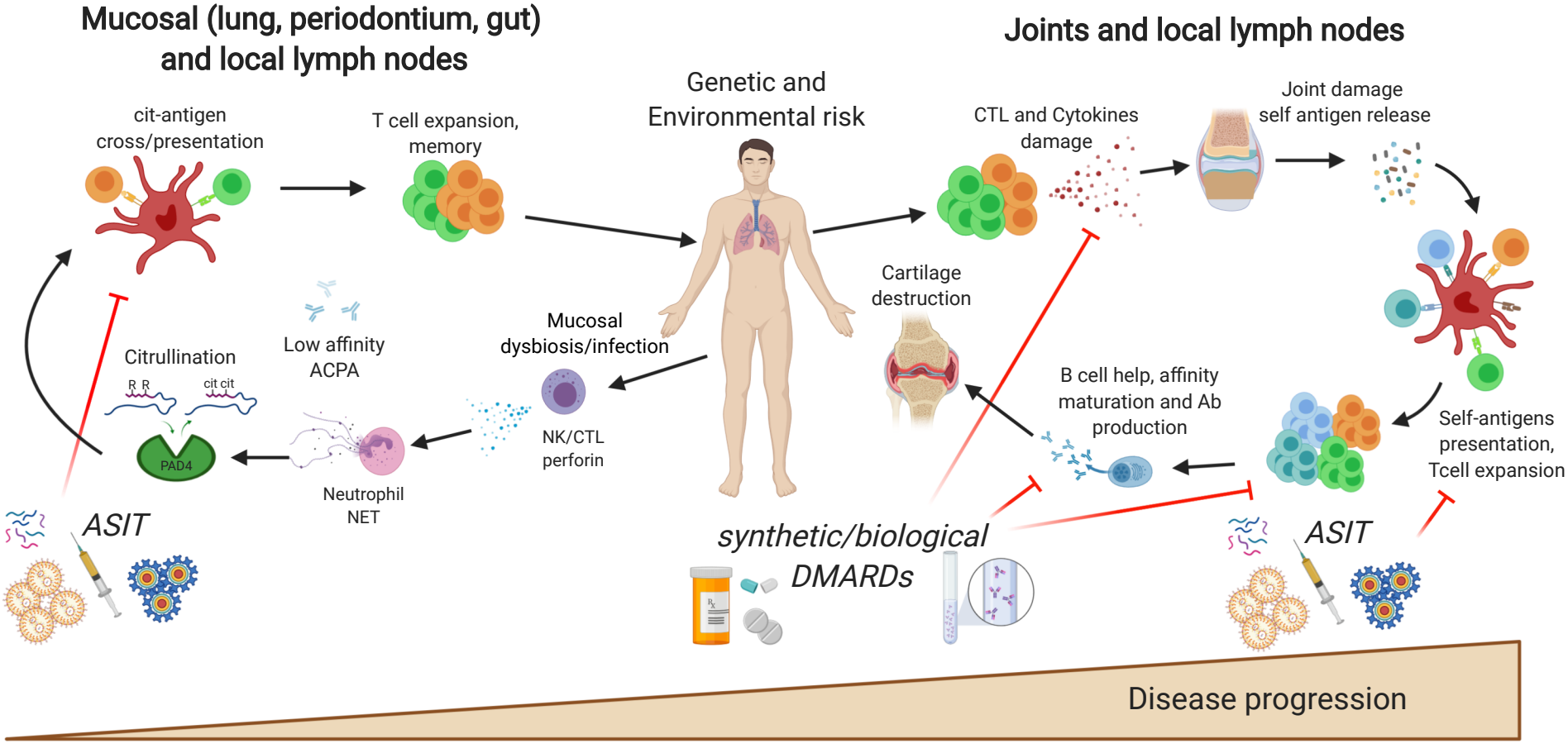
Figure 2. Immunotherapy-induced bystander immune regulation. Immunotherapy with a single, strong epitope induces antigen-specific Treg in draining lymph nodes, which mediate suppressive effects on DC draining inflammatory sites, and decrease T cell activation towards locally-derived tissue-antigens.

Figure 3 Model for the development of autoimmunity through mucosal infection challenge, and antibody-mediated inflammatory arthritis in RA

RA develops through the interplay of autoantigen liberation, presentation, auto reactive CD4 and CD8 T cell expansion, autoantibody development, affinity maturation, and antibody-driven innate inflammation. ASIT: antigen-specific immunotherapy. All figures created with BioRender.com


Mature/Immunogenic DC
MHC class II
CD40
CD80
CD83
CD86
IL12
TNFα
IL1β
IL6
Effector T cells
Th1/2/17
Cytotoxic CD8
Foreign antigens
NF-κB
NF-κB
Self antigens
PD-L1
IDO
TGFβ
IL12
Tolerogenic DC
Regulatory T cells
Treg, Tr1


Distal antigen-specific tolerising immunotherapy
DC cross/presenting multiple antigenic epitopes
Therapy-induced antigen-specific regulatory CD4+ T cells
Joint self-antigens
Joint tissue and draining lymph nodes
Auto-reactive CD4+ and CD8+ T cells


Mucosal (lung, periodontium, gut) and local lymph nodes
Joints and local lymph nodes
Genetic and Environmental risk
cit-antigen cross/presentation
T cell expansion, memory
CTL and Cytokines damage
Joint damage self antigen release
Cartilage destruction
Mucosal dysbiosis/infection
Low affinity ACPA
Citrullination
R R
cit cit
PAD4
NK/CTL perforin
Neutrophil NET
B cell help, affinity maturation and Ab production
Self-antigens presentation, Tcell expansion
ASIT
synthetic/biological DMARDs
ASIT
Disease progression

Appendix 1: Autoantigenic epitopes in RA

Antigen	Source	Animal models, and of tolerance	Clinical trials	Epitopes
Citrullinated antigens		No		
Aggrecan/ proteoglycan	Cartilage	Yes (PGIA) + tolerance		Aggrecan$_{89\text{-}103}$Cit 93, 95 Aggrecan$_{89\text{-}103}$ Cit93,95(G92Y) Aggrecan$_{225\text{-}244}$Cit231,236 Aggrecan$_{553\text{-}570}$Cit556, 561
Vimentin	FLS, NETs		DC	Vimentin$_{59\text{-}71}$Cit64,69,71 Vimentin$_{66\text{-}78}$Cit 71 Vimentin$_{419\text{-}421}$Cit 424
Fibrinogen	ECM		DC	Fibrinogen$\beta_{69\text{-}81}$Cit 72,74 Fibrinogen$\alpha_{79\text{-}81}$Cit 84
α-Enolase	NETs			α-enolase$_{4\text{-}16}$Cit 9, 15 α-enolase$_{11\text{-}25}$Cit 15 α-enolase$_{26\text{-}40}$Cit 32 α-enolase$_{326\text{-}340}$Cit 327
Collagen II	Cartilage		DC	Collagen II$_{1237\text{-}1249}$Cit 1240
Histones	NETs			Histone H2B$_{68\text{-}82}$Cit 73,80 Histone H4$_{33\text{-}47}$Cit 39 binds ACPA
Cartilage intermediate-layer protein (CILP)	Cartilage	No	No	CILP$_{297\text{-}311}$ CILP$_{297\text{-}311}$Cit 305 CILP$_{982\text{-}996}$ CILP$_{982\text{-}996}$Cit 988,991
Collagen II		Yes + tolerance	Oral protein	Collagen II$_{259\text{-}273}$
Heat Shock proteins and chaperones		Yes immuno-modulation		
HSP dnaJ	*E.coli*		Oral peptide(36)	*E.coli* dnaJ-QKRAAYDQYGHAAFE
HSP65	Mycobacteria		ND	HP-R1-QKRAAQDAAVDAACG HP-R2-QKRAAQAARVEAACG HP-R3- QKLFKTLQSLFADFN
HSP60	Stressed cells		s.c. peptides (for T1D)	Hum HSP60$_{437\text{-}460}$C442V; C447V Hum HSP60$_{280\text{-}294}$ Myc HSP60$_{216\text{-}230}$
HSP70			ND	Myco B29 Hum mB29b
HSP4 (BiP)			i.v. protein(37)	BiP$_{336\text{-}355}$ BiP$_{456\text{-}475}$

HSP10 (Chaperone 10)			i.v. protein	ND
Calreticulin (CRT), Citrullinated CRT	FLS	No		Serum, synovial antibody binding, binds HLA-DRB1*0401$_{65\text{-}79}$
Human cartilage glycoprotein 39	Secreted from cartilage with joint inflammation	No	i.n. protein i.v. MHCII:HC gp-39 peptide complex	HC gp39$_{263\text{-}275}$
33kDa A2 proteins		Yes (pristane), no tolerance	Yes	hnRNP-A2$_{117\text{-}133}$ hnRNP-A2$_{120\text{-}133}$ hnRNP-A2$_{117\text{-}133}$Cit 117
N-acetylglucosamine-6-sulfatase (GNS)	SF	No	No	GNS$_{222\text{-}235}$
filamin A	Platelets	No	No	FLNA$_{2446\text{-}2460}$

ND = not done ; iv = intravenous; sc = subcutaneous; in = intranasal

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The autoimmune response and clinical stages preceding the development of Rheumatoid Arthritis.

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Abstract

Rheumatoid Arthritis (RA) is a chronic inflammatory disease affecting synovial joints. Although treatment options and treatment efficacy have increased significantly in the last two decades, the disease cannot be cured or prevented. Therefore, RA still has a considerable impact on the quality of life of patients, not only because life-long medication is often required but also because residual disease activity leads to progressive loss of function in the musculoskeletal system and extra-articular morbidity. Key future goals in the management of RA are the ability to induce long-lasting drug-free remission in patients who have developed RA (i.e. to achieve a cure) as well as to prevent disease before it emerges in the first place. To reach these goals, it is pivotal to understand the autoimmune response underlying RA-pathogenesis and to develop ways to permanently silence it (i.e. to induce tolerance). For preventive studies, the identification of markers (of either clinical or immunological/biological origin) predictive of future disease is crucial, as prevention of disease will not be feasible without the identification of relevant 'at risk' target populations. Here, we will review from a clinical perspective what is known about the "pre-RA-state" and the different clinical phases that can be identified during the transition from health to RA as well as from an immunological angle, the auto-immune response underlying RA, how RA-specific auto-immunity could develop and how "tolerance studies" could be designed to achieve prevention and/or cure of disease.

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by inflammation of synovial joints. At present, the disease is incurable in most patients, necessitating life-long medication. Although, clinical signs and symptoms can be controlled in the majority of patients, current evidence suggests that the underlying autoimmune response is not substantially affected by these treatments[1,2]. On the other hand, it is tempting to speculate that long term drug-free remission could be induced with future treatments aimed at silencing and/or tolerizing the autoimmune reaction that plays a role in RA pathogenesis. In order to design such treatments, it is crucial to understand the immune components contributing to disease. T-cells contribute to RA pathology, likely play an important role in the early stages of disease, and are a rational target for tolerance-induction approaches[3,4]. Such approaches could aim to delete/silence the T-cell population directly responsible for disease-induction and/or progression. Alternative approaches include the induction of a new T-cell population able to steer the autoimmune response in a non-pathogenic/non-inflammatory direction[5]. While in many preclinical animal studies, pre-emptive silencing of the immune response can be readily achieved, silencing of ongoing immune responses in humans is much more challenging[4]. This is, most likely, not only a consequence of the long-standing presence of an autoimmune response which started developing years before the onset of clinical symptoms, but also because the pathogenic T-cell population in humans is often ill-defined and variable between different individuals, even when

affected by the same disease. Likewise, methods for the induction of effective T regulatory cell populations that can modulate disease outcomes in an antigen-specific manner in humans are not currently available. Whilst first successes in type I diabetes have been reported using an anti-CD3-blocking antibody[6], the relevance of these approaches to other auto-immune diseases remain to be elucidated. Here, we will provide an overview of the autoimmune response in RA in relation to the different stages preceding disease-development and the possibilities to modulate this response to prevent disease-onset or disease-progression.

The transition from health to RA

During the transition from health to RA, a number of clinically apparent 'pre-RA' phases can be identified[7]. Initially an asymptomatic subject may develop musculoskeletal symptoms suggestive of underlying joint inflammation (e.g. joint pain and morning stiffness) but in the absence of clinically apparent joint swelling – a phase recently termed Clinically Suspect Arthralgia (CSA)[8]. Patients with CSA may then develop clinically apparent synovial swelling not fulfilling classification criteria for RA – i.e. an unclassified arthritis (UA). Such patients may subsequently progress to RA. However, not all patients who develop RA progress through these phases in this way. For example, some patients' initial symptom onset may manifest as UA without a preceding CSA phase and for others it may manifest initially as RA without a preceding UA phase. Likewise, not all subjects with CSA or UA will develop RA as symptoms might also resolve spontaneously. Nonetheless, in those individuals in whom they occur, these clinically apparent 'pre-RA' phases represent important windows in which therapeutic intervention can be applied to limit the rate of progression to RA[9]. The design of such interventions should be informed by an understanding of the evolution of the RA associated autoimmune response from the asymptomatic state through to, where relevant, the development of CSA and UA, to eventual RA. Such an understanding might be used to develop actionable biomarkers and also to design (patient-tailored) interventions aiming to specifically target and halt the disease-associated autoimmune response.

Predicting transition to RA

In addition to understanding the development and evolution of the autoimmune reaction, it is crucial to develop accurate prediction models in the auto-immune disease field. Without such models, prevention will be difficult to achieve as prediction is vital to identify relevant target populations for prevention approaches. This is not only important for willingness of (pre)patients to accept medications and/or life-style changes[10,11], but also for the design of trials to assess the effectiveness of interventions. Prediction models including various combinations of clinical and serological biomarkers, have been developed[12,13] and perform reasonably well, especially in patients with early joint complaints. Refinement of these models is ongoing and the addition of imaging related variables, together with other biomarkers, may improve performance[14-17]. At present, several clinical trials are underway aiming to prevent development of chronic arthritis in subjects at risk[18-22]. The outcomes of these trials are expected over the next five years and are likely to offer new insights into the design of further interventions in the pre-RA phases aiming to silence the autoimmune response in the long term.

The autoimmune response in RA.

Auto-antibodies are detected in at least half of RA patients at the time of diagnosis. In cohorts of patients with longstanding active disease, the proportion of seropositive patients increases because longstanding drug-free remission is more common in seronegative patients[23]. These observations are most likely a reflection of the fact that RA is a heterogeneous disease consisting of different endotypes with distinct pathobiological mechanisms driving disease induction and progression. Seronegative RA lacks most typical hallmarks defining an autoimmune disease as no disease-specific auto-immune response has been identified, no strong association with the Human Leucocyte Antigen (HLA)-system is detected and whole genome-wide association studies (GWAs) have not revealed consistent associations with genetic regions involved in controlling the adaptive immune response[24-27]. Therefore, the immunological component contributing to seronegative RA is most likely mediated by innate immune responses, rather than adaptive immune responses. The nature of these responses are still ill-defined and it is unclear which cells or which triggers drive the inflammatory response in seronegative RA-patients. The insights that RA comprises at least two different endotypes are important for efforts aiming to silence the pathoimmunological mechanisms underlying these two different disease entities in a long-lasting, preferably permanent manner as they likely require different approaches and treatment regimens.

In contrast to seronegative RA, the immune responses most likely contributing to seropositive RA are better defined. Seropositive RA is, by definition, characterized by the presence of auto-antibodies. Rheumatoid factors (RF)- and anti-Citrullinated Protein Antibody (ACPA)-responses have been studied in most depth and the presence of these antibodies is relevant for disease classification and prognostication[28].

Rheumatoid factors were first identified in the 1940's and recognize the Fragment crystallizable (Fc)-part of human IgGs. They were discovered by the finding that sera from RA-patients could agglutinate red bloods cells coated with IgG from sheep, via RF mediated cross linking of Fc-tails[29,30]. These findings indicated that RFs can bind to immune-complexed antigens, i.e. antigens bound by antigen-specific antibodies forming antigen-antibody complexes. Most likely, the binding of antigen by IgG induces a conformational change, which exposes RF-epitopes on the Fc-tail of the antigen-binding IgGs for recognition by RFs[31,32]. In doing so, RFs can form larger immune complexes, thereby, conceivably, enhancing and exacerbating inflammatory responses through additional recruitment of Fc-receptor- and complement system-mediated effector mechanisms. It is hypothesized that RFs contribute to the pathogenesis of RA via this mechanism[33-35].

The molecular identity of the antigens recognized by ACPA was defined 20 years ago in studies addressing the molecular identity of the antigens recognized by the RA-specific antibodies that had been termed anti-keratine antibodies or anti-perinuclear factors. In these studies, citrulline, an amino acid formed by the post-translational modification (PTM) of arginine, was identified as an essential constituent of the antigens recognized by anti-perinuclear factors/anti-keratine antibodies[36]. ACPA display a higher specificity for RA than do RFs and can be identified by commercially available tests using cyclic citrullinated peptides (CCP) or a model protein antigen, mutated citrullinated vimentin (MCV)[37]. For these reasons, ACPA are also called anti-CCP- or anti-MCV-antibodies. Although the underlying mechanism is unclear, it is likely that either ACPA and/or the underlying B- and T-cell responses directly contribute to the pathogenesis of ACPA-positive RA[38]. For example, the most prominent genetic risk factor for RA encoded by the HLA-region specifically predisposes to ACPA-positive disease but not to ACPA-negative or "RF-only positive" disease[25]. Likewise, one of the RA-susceptibility loci identified by genome-wide association studies in the last decade encodes for peptidylarginine-deiminase, the enzyme responsible for the post-translational conversion of arginine into citrulline[39]. Thus, the specificity of the ACPA-response for RA, combined with the observations that the genetic region encoding the enzymes creating antigens recognized by these antibodies, as well as the finding that the most prominent genetic risk factor, the HLA-region, specifically predisposes to ACPA-positive disease, make it highly likley that the citrulline-reactive immune response (antibodies, T-cells and/or B-cells), is involved in disease pathogenesis. Indeed, since the discovery of

citrulline as antigenic target for ACPA, several lines of evidence support the notion that citrullinated protein-reactive immune responses contribute to (the onset of) the signs and symptoms of RA (reviewed in [25,40-42]).

The progress made in the understanding of the ACPA-response in the last two decades has also led to the realization that the ACPA-response is diverse and targets a plethora of citrullinated antigens. The latter is explained by the observation that ACPA are cross-reactive to many different citrullinated proteins, of self- and non-self-origins, both at the polyclonal as well as the monoclonal level[43-45]. These findings are important for approaches aiming to silence, or "tolerize", citrullinated protein-directed immune responses as it is challenging to define the antigen(s) responsible for inducing, sustaining or propagating anti-citrullinated protein immune responses. This is likely to be even more the case in the context of the T-cell response underlying the citrullinated protein-directed B-cell response, as this T-cell response does not have to recognize citrullinated (self)epitopes. For example, because ACPA-expressing B-cells can recognize multiple citrullinated antigens, it is likely that they are cross-reactive to both citrullinated self- and microbe-derived proteins. Consequently, an ACPA-expressing B-cell could attract T-helper cell-activity, required for its growth and differentiation, by recruiting a T-cell response directed against a microbe-derived antigen that has become citrullinated by e.g. the release of peptidyl arginine deiminases from netosing neutrophils attacking the invading microbe. In this scenario, the responding T-cells are not auto-reactive and could recognize a non-citrullinated epitope that is presented by ACPA-expressing B-cells recognizing a particular citrullinated protein. Hence, the autoreactive B-cell response does not have to be supported by an autoreactive T-cell reaction.

More recently, it has been shown that ACPA not only recognize citrullinated antigens, but that they can also interact with carbamylated and acetylated proteins, adding another dimension to the complexity of the antigens targeted by the RA-specific auto-immune response[46,47]. Importantly, these discoveries are highly relevant for the conception of strategies to silence the T-cell response underlying RA in an antigen-specific manner using defined antigens, as they indicate that success in such approaches is, at present, likely difficult to accomplish. This notion is mainly based upon the findings that the pool of possible candidate T-cell antigens amenable for targeting seems large and diverse, making it very challenging to select the appropriate T-cell targets for efficacious targeting of the underlying T-cell response which sustains and "helps" the ACPA B-cell response in a given patient.

The evolution of the RA-associated autoimmune response.

The discovery of the auto-antibody responses characteristic of seropositive RA also provided fresh encouragement for studies unravelling the induction and evolution of these auto-immune responses. It is now clear that both the RF- and ACPA-response can be present years before subjects develop RA[48-51]. Recent evidence indicates that both RF and ACPA can also be present in unaffected healthy individuals without progression to RA. In these individuals, the auto-antibody response can stay at relatively low level for years, and can even disappear over time[52]. The latter observations are interesting as they suggest that these auto-immune responses are part of conventional immune responses against microbes encountered by the immune system. In contrast, the ACPA- and RF-response do not disappear in subjects that transition to RA. Instead, several lines of evidence indicate that the auto-immune response undergoes an expansion before the development of RA. For example, isotype-usage, autoantibody-levels and the citrullinated epitope recognition profile of the ACPA-response increase before onset of RA[53-56]. Nonetheless, although generally accepted that the broadening of the auto-immune response takes place before the onset of RA, it is less well defined in which pre-RA phase the expansion of auto-immunity occurs. In some individuals, high levels of ACPA can be found years before disease-onset in the absence of apparent musculoskeletal symptoms, whereas in others a broadening of auto-immune responses is found relatively close to the time of joint

swelling[48,49,52]. Intriguingly, a rather small increase in the avidity of the ACPA-response is observed over time, indicating that isotype-switching and avidity maturation are uncoupled in this auto-immune response[57,58]. Instead, it has been proposed that another feature of ACPA, the acquisition of N-linked glycans in the variable domain, is involved in the maturation and expansion of the ACPA-response[42,59,60]. Such glycans are absent from most antibodies, but abundantly present on ACPA from RA patients, making it a unique feature of the RA-specific ACPA-response[61]. In contrast, however, in ACPA-positive healthy individuals that do not transition to RA, no such glycans are found, suggesting that the presence of these glycans could represent a marker for a "healthy" and an "unhealthy" ACPA-response. Indeed, in a cohort of first degree individuals (FDRs) from RA-patients it was found that FDRs who later developed RA showed extensive variable domain glycosylation before the onset of arthritis and that IgG ACPA variable domain glycosylation was strongly associated with future development of RA[60]. As the acquisition of variable domain glycans results from a selective introduction of N-linked glycosylation-sites by somatic hypermutation, it is tempting to speculate that the acquisition of variable domain-glycans by ACPA-producing B-cells allows the B-cell response to expand, thereby contributing to precipitation of disease. In this respect, it is noteworthy that ACPA-IgG variable domain glycosylation increases closer to symptom onset and associates with anti-CCP2 antibody levels pre-disease, but not after disease onset, in line with the notion that these glycans facilitate the expansion of the ACPA-response[59]. Nonetheless, recent data obtained by analysing pre-RA samples also indicate that ACPA variable domain glycosylation can take place years before RA-development, suggesting that additional biomarkers are needed to describe the CSA- and UA pre-RA-phases in immunological terms[59,60].

The induction of the RA-associated autoimmune response.

The initial antigenic drivers of the induction of the auto-immune response are not known, but it is widely held that these may be microbial components in mucosal tissues that could activate autoreactive B-cells[62-69]. As many autoreactive B-cell responses are isotype-switched and are found long before disease onset, these B-cells must have received T-cell help – potentially from microbe-specific T-cells. As most microbe-directed T-cell responses are broad and present in all healthy individuals, it is unlikely that this T-cell response is restricted by only a few HLA-alleles. Indeed, the ACPA IgG-response as found in unaffected healthy individuals does not appear to associate with the presence of the specific HLA-alleles predisposing to RA, pointing to the presence of extensive T-cell responses restricted by many different HLA-molecules[70,71]. Therefore, it is likely that the B-cells fuelling the initial ACPA-response receive helper activity from T-cell responses that are not restricted to the predisposing HLA-molecules, but to other HLA-molecules as well. Instead, other T-cells, restricted to the HLA-molecules predisposing to RA, are likely involved in the subsequent expansion of the initial ACPA-response occurring before disease onset as ACPA-positive RA is hallmarked by a clear association with defined HLA-molecules. Thus, current evidence indicates that different T-cell responses underlie the initial induction versus, respectively, the expansion of the ACPA-response. At present, the identity of the antigens recognized by the T-cells responsible for either the initial induction or the "second expansion" of the ACPA-response is unknown and could, potentially, be of self- and non-self-origin.

Similarly, the induction of RF-specific immune responses has been postulated to involve T-cells which recognise microbial antigens[72]. Many microbes are recognized by conventional antibodies and the interaction can lead to microbe-IgG immune complexes. RF-expressing B-cells could recognize such immune complexes. This could not only lead to the direct activation of these B-cells by toll-like receptors[73], but also to the concurrent recruitment of microbe-directed T-cell help for their further

maturation and expansion. In case these B-cells or the RFs they produce interact with ACPA complexed with citrullinated self-antigens, they could further contribute to the inflammatory response in for example the inflamed synovium.

Is there tolerance in the pre-RA-state and could tolerance be induced?

As indicated above, the antigens recognized by the T-cells involved in the auto-immune response underlying RA have not been well defined. As it is possible that these antigens are different in different patients and can fluctuate over time within a patient during disease as well as in different pre-RA-phases, it might prove very challenging to design T-cell targeted antigen-specific approaches to silence inflammation in RA. Current data also suggest that in the pre-RA-state, T-cell tolerance to the antigens recognized by ACPA-expressing B-cells is likely not present as isotype switched and somatically mutated ACPA derived from "helped" B-cells can appear years before disease-onset[59]. Therefore, it might proof difficult to design ways to "maintain" a tolerant state before disease onset as this state might have disappeared long before. However, given that the citrullinated protein-directed immune response is dynamic and continuously active[74,75], approaches to modulate the activity of those antigen-presenting cells required to induce and steer T-cell responses, might prove more rewarding. An advantage of in vivo modulation of dendritic cells is that the identity of the antigens presented by the body to sustain and expand the autoimmune response can remain unknown. Approaches focussing on antigen-presenting cells could, for example, entail targeting of immunosuppressive agents by dendritic cell-directed liposomes or nanobodies[76,77]. Indeed, in the field of tumor-immunology several approaches to deliver therapeutics specifically to dendritic cells are under development[78,79]. Likewise, also in the autoimmune disease field such methods are being explored. For example, calcitriol-containing liposomes have been shown to modulate human dendritic cells phenotype and function and could, potentially, be used to regulate/dampen ongoing auto-reactive T-cell responses that undergo recurrent activation by dendritic cells[80]. Whether continual reactivation of disease-contributing T-cell responses is occurring in established RA needs to be established, but current evidence indicates that this is an plausible option. Similarly, such information is currently not available for pre-RA-phases and needs to be obtained. However, given the expansion and maturation of the auto-antibody response before the onset of arthritis, it is likely that in this phase RA-related T- and B-cell responses, are active and expanding. For this reason this might be a preferred phase for interventions aiming to tolerize and/or silence RA-related auto-immune responses using antigen-independent methodologies. Such interventions could aim to specifically inhibit ongoing T-cell responses by pharmacological modulation of dendritic cells, but might also entail other approaches such as targeting T-cells by anti-CD3 antibodies. Likewise, also other cells involved in disease pathogenesis could be targeted in a specific manner including B-cells and potentially even fibroblast-like synoviocytes that are thought to provide the "niche" in which the deranged auto-immune response is embedded[81,82].

Another appealing approach to modulate the RA-associated immune response, because of simplicity and safety, might be dietary modification to modulate the microbiome. Multiple microbial species present in the gut-microbiome can produce short-chain fatty acids that possess immunomodulatory activity. For example, microbe-produced short chain fatty acids, such as butyrate have been shown to modulate T-cell differentiation, cytokine-production and promotion of peripheral regulatory T-cell generation[83,84]. As the production of such metabolites by gut bacteria could, potentially, be modulated by certain dietary interventions[85], it would be attractive to investigate their impact on the RA-associated autoimmune response in e.g. different "pre-RA" phases combined with the evaluation of the progression/decline of symptoms to the "RA-phase"[86,87].

Although the road ahead appears long and challenging, the unprecedented progress made in our understanding of the clinical- and immunological stages preceding disease-onset, combined with the

first successes with tolerizing interventions in other autoimmune diseases offers great hope for the development of a cure for this chronic disease by specific silencing of the underlying auto-immune response.

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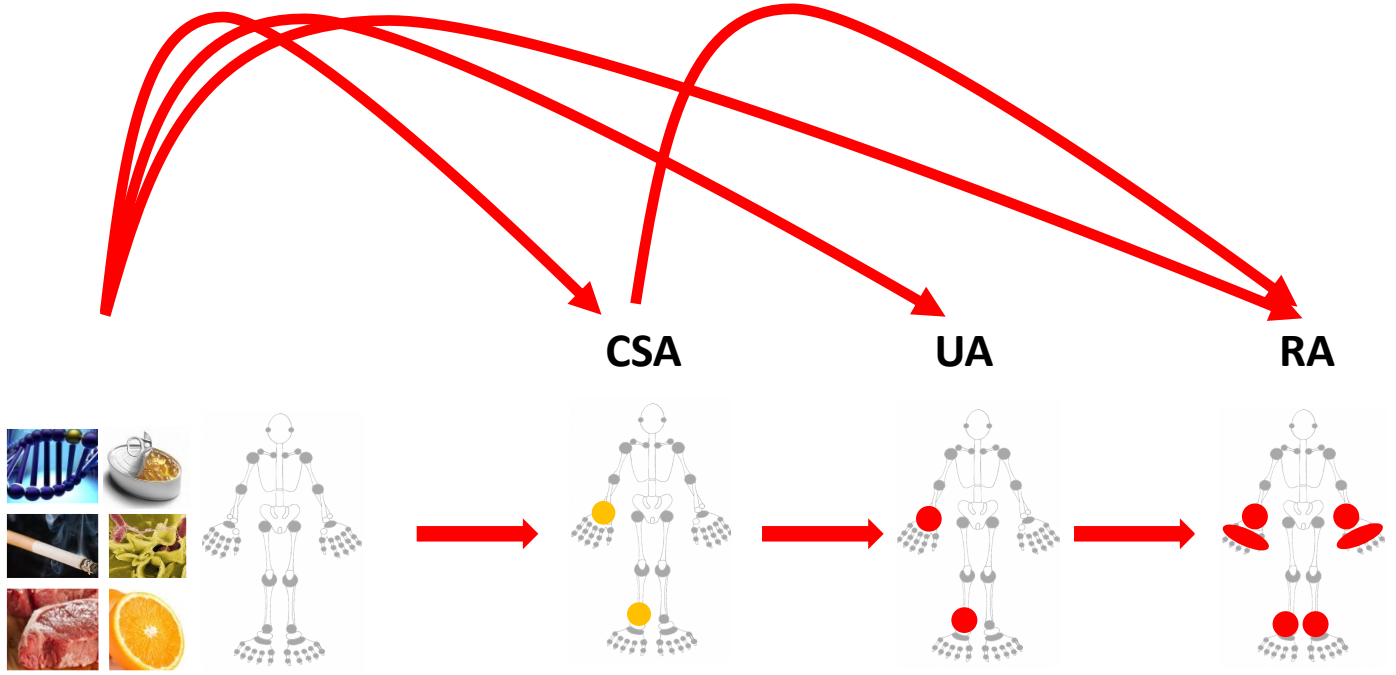
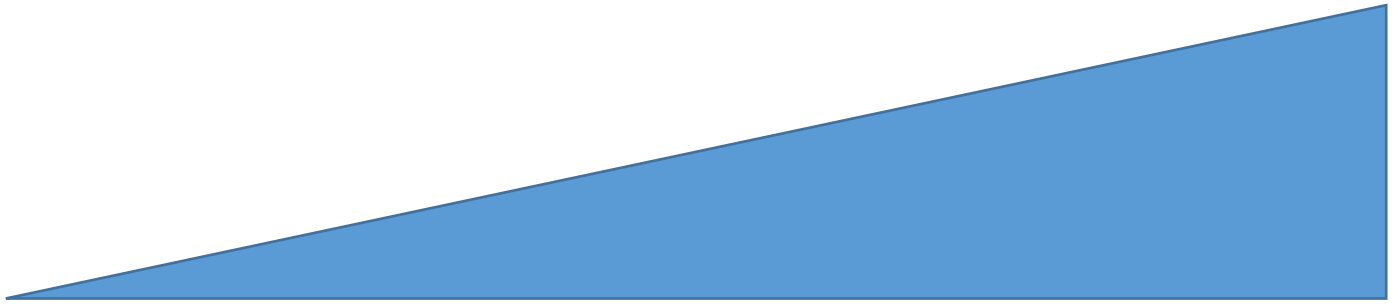
Legends

Figure 1: Graphical depiction of the stepwise evolution of autoimmune response in RA related to progression from one clinical phase to another.

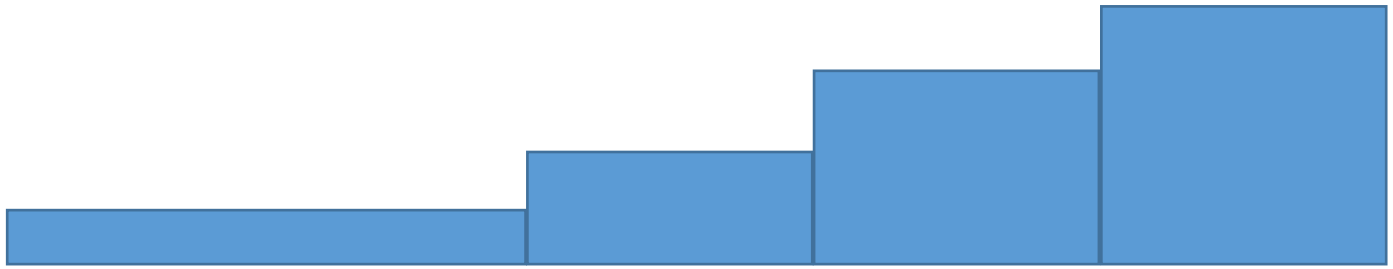
Figure 2: Graphical depiction of how the anti-citrullinated protein antibody (ACPA) immune response is hypothesized to emerge and progress in time till development of rheumatoid arthritis (RA). It is postulated that the initial trigger for the induction of the autoimmune response is derived from microbes. This induction is independent of the HLA-molecules predisposing to RA. Subsequent events of unknown origin leads to the expansion of the ACPA-response and the introduction of variable domain glycans by ACPA-expressing B-cells. Current evidence indicates that the expansion of the ACPA-response is associated with the HLA-molecules predisposing to RA and likely involves a 2nd involvement of T-cells, independent from the T-cell response implicated in the initial induction of the ACPA-response. Key future goals in the management of RA are the ability to induce long-lasting drug-free remission in patients who have developed RA (i.e. to achieve a cure) as well as to prevent disease before it emerges in the first place. This could, potentially, be achieved by silencing/tolerizing the autoimmune response underlying RA.



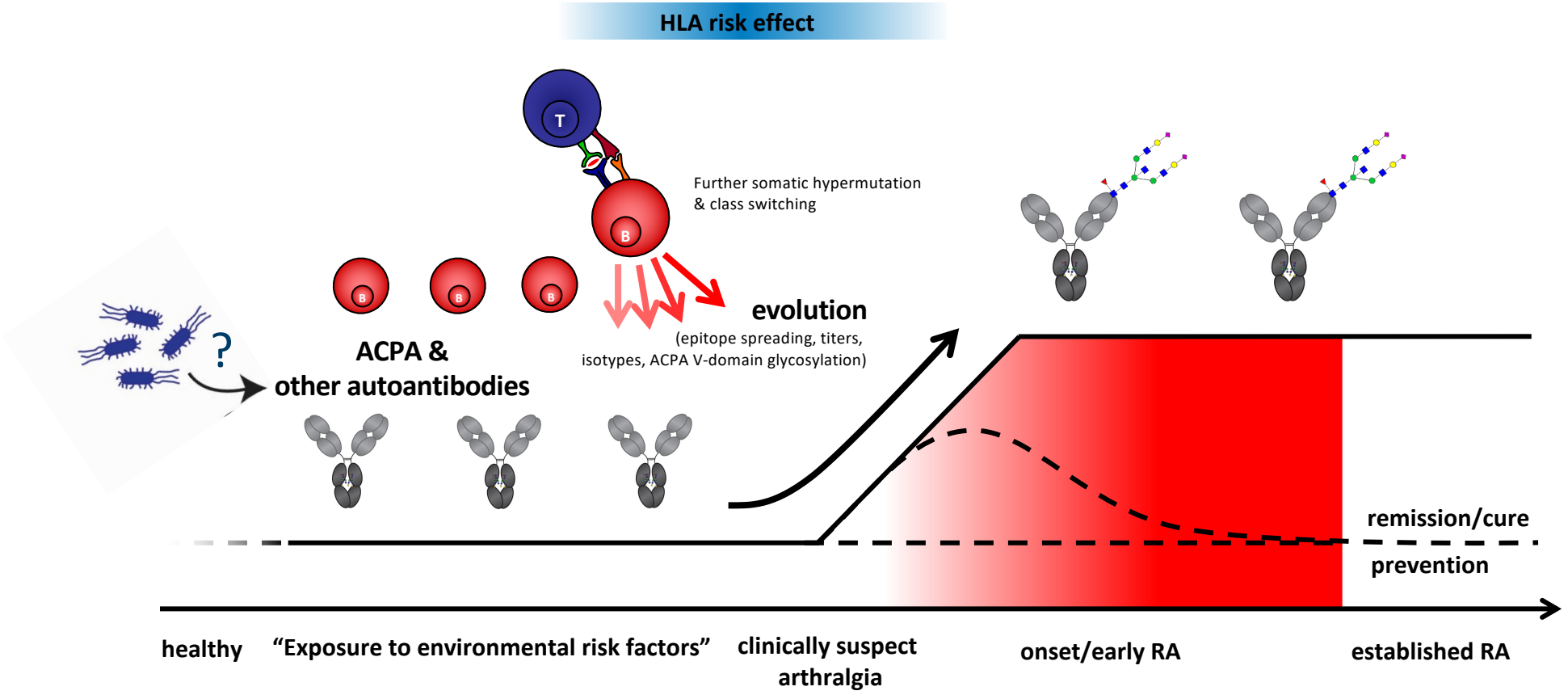
Gradually evolving autoimmune response



Stepwise evolution of autoimmune response related to progression from one clinical phase to another



Dynamics of RA and of the ACPA response



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Biomarkers of tolerance in immune-mediated inflammatory diseases: a new era in clinical management?

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Biomarkers of tolerance in immune-mediated inflammatory diseases: a new era in clinical management?

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Abstract

Modern therapeutic agents and treatment regimens have made sustained remission an achievable target for many patients across a spectrum of immune-mediated inflammatory diseases, albeit at the risk of adverse events and the expense of drug prescription and safety monitoring. Clinicians and patients are thus increasingly faced with a novel treatment dilemma – whether and how best to stop immunomodulatory treatment in patients who achieve remission. In this final paper in a Series on therapeutic tolerance induction, we summarise our current knowledge of biomarkers of immune homeostasis in human immune-mediated inflammatory diseases and their application to the prediction and achievement of sustained drug-free remission. We summarise evidence from prospective studies of immunomodulatory drug cessation across a range of immune-mediated inflammatory diseases, including rheumatoid arthritis, juvenile idiopathic arthritis, and inflammatory bowel disease, and we consider current evidence for clinical, serologic, proteomic, metabolomic, cellular, and microbiome biomarkers of immune homeostasis. The steps necessary for clinical translation, and the potential transformative effect of these biomarkers on management of patients with immune-mediated inflammatory diseases if successfully achieved, are discussed.

Introduction

The past two decades have witnessed a remarkable revolution in the management of immune mediated inflammatory diseases. Whereas progressive end organ damage, disability, and mortality were previously viewed as inevitable consequences of unrelenting chronic inflammation, sustained remission is now achievable in substantial proportions of patients across a range of different diseases. These impressive advances have been achieved largely through a combination of early diagnosis combined with treat-to-target strategies utilising an increasingly broad armamentarium of immunomodulatory agents—a therapeutic blueprint that has been successfully replicated across a wide spectrum of immune mediated inflammatory diseases.[1-5] A prime example is in rheumatoid arthritis (RA), where management has shifted from a historical approach of symptom palliation with non-steroidal anti-inflammatory drugs and glucocorticoids to rapid and tight control of disease activity, with 50-60% of patients now achieving clinical remission (as defined by DAS28) in modern cohort studies.[6,7]

Nevertheless, the increasing use of potent immunomodulatory agents in the treatment of immune mediated inflammatory diseases poses challenges in terms of risk of medication-related adverse events,[8] the resources required for regular safety monitoring,[9] and the substantial prescription costs of newer biologic and targeted therapies. These drawbacks provide impetus for the development of novel strategies of treatment de-escalation and cessation, with consequent benefits for both patients and healthcare systems. Central to this step-down therapeutic approach is an ability to define and quantify the mechanisms underlying the restoration of immune homeostasis in order to identify those patients most likely to benefit.

In this Series paper, we summarise recent developments in the search for biomarkers of immune homeostasis in human immune-mediated inflammatory diseases, from early work exploring immune tolerance biomarkers in solid organ transplantation to recent clinical trials of drug cessation in the setting of remission in patients with immune-mediated inflammatory diseases. Finally, we discuss

the key steps required to translate such biomarkers to the clinic, and the potential transformative impact on disease management pathways if this is successfully achieved.

Operational tolerance - early lessons from solid organ transplantation

Early insights into the nature of human immune tolerance biomarkers were provided by pioneering studies in solid organ transplantation. Combinations of potent immunosuppressive drugs are prescribed to recipients of solid organ allografts to prevent immune-mediated rejection, and these drugs are then usually continued for the duration of graft survival, often life-long. Occasionally immunosuppression is stopped, sometimes because of life-threatening toxicity but more commonly because of patient non-adherence. In most cases this results in rejection of the allograft, but in rare cases the graft is not rejected and the immune responses against infections and other foreign antigens is preserved—a state known as operational tolerance.[10]

Several international consortia have explored potential biomarkers of operational tolerance, providing unique insights into this dramatic manifestation of human immune homeostasis. Cohort studies have revealed distinct whole-blood gene expression signatures uniquely associated with operational tolerance. Intriguingly, these transcriptomic signatures differ between different organ grafts: operational tolerance in renal transplant recipients is characterised by expression of genes involved in B cell function,[11] whereas in liver transplantation a signature enriched in NK-cell genes is observed.[12] The relative ease of liver tissue biopsy combined with a greater propensity for operational tolerance has facilitated the prospective study of immunosuppressive drug withdrawal in patients undergoing liver transplantation. In a trial of 75 patients with stable liver graft function on minimal immunosuppression, [13] 33 (44%) patients successfully achieved operational tolerance at 12 months after drug cessation. Operational tolerance was associated with hepatocyte expression of iron homeostasis genes at the start of tapering, and this association was validated in a further cohort of 55 patients.[13]

Although these studies included small numbers of participants, they provide important proof-of-concept evidence to support the feasibility of identifying biomarkers of tolerance in humans. While such biomarkers have potential to help identify those patients likely to benefit from withdrawal of immunosuppression, they may not necessarily help to define the underlying immune processes involved, and they might not relate to antigen-specific mechanisms. Nevertheless, the distinct transcriptomic signatures observed in renal and liver transplantation raise the possibility of distinct pathways to immune tolerance, specific to the anatomical and pathophysiological context.

Drug-free remission as a model of tolerance

With increasing numbers of patients with immune-mediated inflammatory diseases achieving sustained disease remission, clinicians and patients are faced with the dilemma of how best to manage long-term immunomodulatory treatments in this setting. Minimisation of drug treatment carries advantages of reduced medication-related adverse events and reduced prescription costs, and drug tapering (though not necessarily complete cessation) is now endorsed by international consensus management guidelines for several immune-mediated inflammatory diseases including

RA,[1] psoriatic arthritis,[4] and inflammatory bowel disease (IBD).[2] Indeed, in some cases it is possible to completely stop all immunomodulatory therapy and maintain a state of drug-free remission–a potential cure akin to operational tolerance in organ transplantation. For example, interventional trials of drug cessation in patients with rheumatoid arthritis have consistently demonstrated that around half of patients who achieve remission with conventional synthetic disease-modifying anti-rheumatic drugs (DMARDs) can maintain remission for sustained periods after complete DMARD withdrawal.[14-17]

At present, whether the immune system of such individuals has returned to health, and the patients are permanently cured, can only be determined by long-term observation. However, attainment of drug-free remission at least means that immune homeostasis has been reinstated in the medium term. Prospective studies of complete drug cessation provide an ideal experimental model by which to explore possible biomarkers of tolerance or reinstated immune homeostasis in patients with immune mediated inflammatory diseases. Such studies have adopted one of two similar, but conceptually distinct, approaches: the identification of biomarkers that predict sustained drug-free remission, or identification of biomarkers that predict relapse of disease (i.e. flare). Although in practice, both approaches define outcomes based on disease activity below or above a predetermined remission threshold, the conceptual implications from an immunopathological viewpoint are worthy of consideration. Drug-free remission could be defined by the absence of immune mediators of flare, comparable to immune tolerance in the healthy state; alternatively, drug-free remission might require the presence of homeostatic mediators that actively maintain a state of remission in the context of disease-associated immune dysregulation, which may or may not reflect the healthy state. The two concepts are not mutually exclusive and, in keeping with known mechanisms of immune tolerance (such as deletional, anergic, and regulatory tolerance), it is probable that drug-free remission in patients with immune-mediated inflammatory diseases is achieved through a balance between reduced flare-promoting and increased remission-permissive factors (**figure 1**). Thus whereas biomarkers of disease flare are conceptually distinct from biomarkers of sustained drug-free remission, they can both be utilised to quantify and predict immune homeostasis (**table**).

Here, we present data from selected studies of drug-free remission as a paradigm for immune homeostasis in immune-mediated inflammatory diseases. We do not discuss data from dose optimisation (i.e. partial tapering) studies, which is outside the scope of this review, unless such studies illustrate potential biomarkers in contexts where complete drug cessation studies do not exist. Although the majority of published data on biomarkers of immune homeostasis pertains to inflammatory arthritis (namely RA and juvenile idiopathic arthritis [JIA]), we also include the limited available data in IBD. Despite the differences in underlying disease pathogenesis, it is possible that immune homeostatic mechanisms overlap and, furthermore, in many instances the same immunomodulatory drugs are used to treat these diseases.

Biomarker studies in immune mediated inflammatory diseases

Disease activity biomarkers

Studies of drug withdrawal in immune mediated inflammatory diseases have identified common clinical characteristics of patients who can successfully achieve sustained drug-free remission. For example, cohort studies of patients with RA (and also IBD) have consistently demonstrated that elevated disease activity scores prior to DMARD cessation are associated with reduced likelihood of subsequent drug-free remission.[16,18-21] These observations support the clinically intuitive notion of deep clinical remission, whereby robust suppression of disease activity is required before drug withdrawal may be considered.[22] Nevertheless, the precise definition of remission in this context remains controversial, and a lack of standardised definitions for remission and flare in drug withdrawal studies, combined with different baseline clinical characteristics, makes it difficult to compare across different cohorts.

To address the lack of standardisation, several studies have explored the utility of more objective measures of disease remission, such as imaging or histological criteria. In various (mainly biologic agent) tapering studies of patients with RA, the presence of ultrasound measures of synovitis (power Doppler signal, alone or combined with greyscale) has been shown to predict future arthritis flare.[23] However, many of these studies included patients with low disease activity, and very few studies have examined the role of musculoskeletal ultrasound in predicting drug-free remission. In a study of 157 patients with RA in sustained clinical remission (DAS28-ESR < 2.6 for > 6 months), no significant association was observed between the presence of greyscale or power Doppler synovitis (or both) at baseline and occurrence of arthritis flare following DMARD tapering or cessation.[24] In the Biomarkers of Remission in Rheumatoid Arthritis (BioRRA) study,[14]44 patients with RA in remission (DAS28-CRP < 2.4 and absence of power Doppler synovitis on a 7 joint ultrasound scan) stopped conventional synthetic DMARDs and were followed for 6 months. 23 (52%) experienced an arthritis flare, with no association observed between baseline ultrasound measures of synovial or tenosynovial greyscale change, or erosions, with subsequent attainment of drug-free remission – however, the predictive utility of baseline power Doppler signal was not assessed.[14] The limitations of musculoskeletal ultrasound in this setting include the potential for substantial inter-observer variability and non-specific low-level ultrasound abnormalities.[25] Furthermore, the negative findings of recent imaging-based treat-to-target trials in RA have shed doubt on the role of imaging modalities (both ultrasound and magnetic resonance imaging) in defining remission.[26-28] Given these limitations, there is currently insufficient evidence to conclude whether imaging adds further useful information beyond clinical measures of disease activity in the prediction of future disease-free remission in patients with RA.

In summary, clinical data from studies of drug-free remission—at least in RA—point to the importance of robust and sustained remission as a clinical state favouring successful drug withdrawal and subsequent drug-free remission. While not measures of tolerogenic immune mechanisms *per se*, clinical measures of disease activity, combined with measures of subclinical inflammation, have an important role in the selection of patients who are more likely to benefit from drug withdrawal strategies. However, precise definitions of such deep remission states in immune-mediated inflammatory diseases remain debateable, as is the relative contribution of clinical versus subclinical disease activity assessments when considering strategies of immunomodulatory drug withdrawal.

Serologic biomarkers

A hallmark of many immune-mediated inflammatory diseases is the presence of autoantibodies, useful as an aid to diagnosis and, in some cases, as a biomarker of disease activity (e.g. double-stranded DNA antibodies in systemic lupus erythematosus).[29] The presence of autoantibodies is clear evidence of breach of self-tolerance, and their utility as biomarkers to predict attainment of drug-free remission has been explored in the context of immune-mediated inflammatory diseases, notably RA.

Approximately 80% of patients with RA are seropositive for rheumatoid factor (RF), anti-citrullinated peptide autoantibodies (ACPA), or both.[30] Patients with seropositive RA are more likely to develop joint erosions and disability compared with their seronegative counterparts,[31] and the presence of ACPA and RF are negatively associated with subsequent attainment of drug-free remission.[15-17,20,24] Indeed, the replication of this finding across multiple studies underscores the importance of seropositivity as a negative predictor of drug-free remission in RA. Other anti-posttranslationally modified protein antibodies (AMPAs), not currently in widespread clinical use, have also been negatively associated with drug-free remission in RA. In the RETRO study, 101 patients with RA in clinical remission were randomised to one of three arms: DMARD continuation, DMARD tapering to half dose, or DMARD tapering followed by complete cessation.[15] In an exploratory analysis of 94 patients, increasing numbers of AMPAs at baseline was associated with increasing risk of disease relapse (18% of patients with 0-1 AMPAs relapsed *vs* 55% of patients with more than 5 AMPAs; p = 0.011).[32] Similar results were observed in an exploratory analysis of the IMPROVED study, a therapeutic strategy trial of methotrexate plus sulfasalazine plus hydroxychloroquine versus methotrexate plus adalimumab, in which drug tapering to complete cessation was permitted when sustained clinical remission was achieved.[33] In an exploratory analysis of 399 seropositive patients, an increasing number of AMPAs at baseline was associated with improved response to treatment but also with lower rates of early drug-free remission (37% of patients with 1-2 AMPAs achieved drug-free remission for 4 months vs 11% in patients with 3 or more AMPAs; $p = 0.005$).[34] However, when using a more stringent definition of long-term drug-free remission of 12 months or longer, maintained to last study visit, no significant association was observed.

These observations have led to the notion of different levels of remission in RA, with so-called immunological remission (i.e. seroreversion from a seropositive to seronegative state) representing true restoration of immune tolerance.[35] Although conceptually appealing, this suggestion has limited clinical utility in view of the relative permanence of autoantibodies in RA and the rarity at which autoantibodies (particularly ACPA) are lost.[36] The specificity of autoantibodies as a biomarker of disease-free remission is also rather limited, as 30-45% of ACPA-positive individuals can still achieve drug-free remission,[16] and in patients who achieve this state, persistence of ACPA or RF (or both) is common. In an observational study of 95 seropositive patients with RA who achieved sustained drug-free remission (median 4.2 years duration), only 12.8% became ACPA seronegative, and 19.7% became RF seronegative; seroreversion did not differ between those who maintained remission and those who relapsed.[37] Similarly, no significant association was seen between relative changes in autoantibody titres (including RF, ACPA and AMPA) following DMARD cessation and the subsequent achievement of drug-free remission by seropositive patients in the IMPROVED study.[38]

Given these limitations, it is unlikely that autoantibody status alone will be sufficient for clinical use as a robust biomarker of drug-free remission; however, study of autoreactive B cells may hold promise. Using a tetramer-based approach, Kristyanto *et al*.[39] identified a distinct phenotype of ACPA+ memory B cells in patients with established RA that were not present in those with pre-RA. These cells—expressing high levels of the costimulatory molecules CD80 and CD86, low levels of the inhibitory receptor CD32, and having a propensity for secretion of the neutrophil chemoattractant interleukin (IL)-8—persisted in patients with established RA after treatment, even in those with low clinical disease activity scores. Such studies of antigen-specific B cell function may thus yield novel biomarkers of immune tolerance mechanisms, although these have yet to be applied to the setting of drug-free remission in immune-mediated inflammatory diseases.

Proteomic biomarkers

The measurement of circulating inflammatory mediators provides a convenient approach to biomarker discovery that is compatible with a wide range of commercially available clinical-grade assays. Individual cytokines and chemokines have demonstrated predictive value in drug-free remission in some settings; for example, circulating IL-6 levels are negatively associated with drug-free remission in patients with RA following withdrawal of the anti-IL-6 agent tocilizumab.[40] Alternatively, several cytokines and chemokines can be combined to give a composite measure of immune activity, such as the so-called multibiomarker disease activity score (MBDA),[41] which combines the measurement of 12 pro-inflammatory cytokines and chemokines in a single score that has been shown to correlate with clinical measures of disease activity in RA[42] and to predict future radiographic progression.[43-45] In the RETRO study, a high MBDA score prior to DMARD withdrawal was associated with a greater risk of flare in a multivariate model (OR 8.5, 95% CI 2.0-36.4, p = 0.004).[46] Furthermore, the combination of baseline MBDA with ACPA status yielded an even greater discrimination for future flare (76% of patients positive for both MBDA and ACPA experienced flare compared with 32% of patients positive for MBDA alone, and 13% of patients negative for both).[46] These results demonstrate that the presence of subclinical inflammation is sufficient, at least in susceptible individuals, to manifest in overt clinical disease relapse upon DMARD withdrawal. In contrast, high MBDA score at the point of diagnosis has been shown to predict higher rates of future drug-free remission in seronegative (but not seropositive) RA, suggesting distinct subgroups of disease distinguished by differing propensities for drug-free remission.[47]

Circulating inflammatory biomarkers have also shown promise in the prediction of drug-free remission in patients with juvenile idiopathic arthritis. In a randomised trial,[48] elevated baseline concentrations of serum calprotectin (a phagocyte activation marker) and S100A12 (a neutrophil activation marker) predicted subsequent disease flare within the first 3 months after methotrexate discontinuation with an area under the receiver operating characteristic curve (ROC_{AUC}) of 0.75 (0.60 - 0.90) for calprotectin and 0.70 (0.53 – 0.87) for S100A12 Serum calprotectin was a strong predictor of arthritis flare at 12 months after methotrexate discontinuation in another study of 22 patients with JIA in remission (ROC_{AUC} 0.95, 95% CI 0.85 – 1.00)[49] and also predicted flare in some studies of patients who did not discontinue treatment,[50,51] but not in others. [52,53]Thus serum calprotectin appears to be a sensitive biomarker of subclinical disease activity in JIA rather than a measure of underlying immune homeostasis.

In a multi-parameter exploratory analysis of the U-ACT-Early study, Teitsma *et al*[54] showed that among 85 inflammatory proteins measured, higher levels of C-C motif chemokine ligand 18 (CCL18), CCL20, and soluble IL2 receptor alpha (sIL2-Rα) were predictive of future drug-free remission when measured prior to initiation of methotrexate, tocilizumab, or both.. However, it is likely that these cytokines and chemokines are predictors of response to treatment rather than biomarkers of immune tolerance mechanisms.

Metabolomic biomarkers

Recent years have witnessed an increasing interest in the application of high-throughput mass spectrometry and nuclear magnetic resonance spectroscopy technologies to measure small molecule metabolites as an indicator of underlying immune processes.[55] If immune homeostasis in immune-mediated inflammatory disease is restored through distinct metabolic processes, it is conceivable that the metabolites produced or consumed by such processes could be utilised as clinical biomarkers.

In an exploratory analysis of the U-Act-Early study, mass spectrometry was used to quantify 263 metabolites in baseline serum samples from 60 patients with early RA prior to treatment with methotrexate, tocilizumab, or both.[56] Network analysis revealed distinct metabolic pathways associated with drug-free remission depending on the treatment arm: arachidonic acid metabolism was associated with tocilizumab monotherapy, arginine and proline metabolism with methotrexate monotherapy, and histidine metabolism with dual therapy. As only pre-treatment samples were obtained, the metabolic pathways identified likely reflect responsiveness to therapy (i.e. theragnostic biomarkers) rather than biomarkers of immune homeostatic processes. Nevertheless, the distinct pathways observed for different treatment arms raises the possibility of medication-specific routes to drug-free remission, even within a common disease.

Cellular biomarkers

All of the methodologies discussed thus far aim to quantify biomarkers in the circulation (whole blood, serum, or plasma). Whereas these approaches are inexpensive and allow for rapid laboratory processing, they may not provide sufficient resolution to identify faint biomarkers signals or biomarkers that are specific to a rare immune cellular compartment. This is further compounded by wide variations in the cellular composition of peripheral blood between different individuals. To address these problems, several research groups have explored cell-type specific biomarkers of disease-free remission (phenotypic or transcriptional) in immune-mediated inflammatory diseases, an approach that offers the potential for richer phenotyping and better resolution of candidate biomarkers at the cellular level.

In the aforementioned BioRRA study,[14] disease flare following cessation of conventional synthetic DMARDs could be predicted by the baseline expression of three genes within circulating CD4+ T cells: *FAM102B*, *AC073343.2*, and *EPS15-AS1*. Although the functional relevance is unclear, a composite score combining expression of these three genes together with baseline concentrations of circulating IL-27 and a clinical measure of disease remission (ACR/EULAR Boolean remission) could

predict future arthritis flare versus remission, with an AUC_{ROC} of 0.96 (95% CI 0.91–1.00); work is ongoing to validate these findings in an independent cohort.

In another exploratory analysis of the U-Act-Early study, distinct CD4+ T cell transcriptomic modules associated with subsequent disease-free remission were observed in the different treatment arms: leukocyte migration and G-protein signalling pathway modules were associated with tocilizumab monotherapy, response to bacteria or biotic stimuli modules with methotrexate monotherapy, and transcription and translation modules with dual therapy. [57]

Subsets of circulating lymphocytes associated with flares have been defined in immune-mediated inflammatory diseases, and several groups have explored whether they are predictive of sustained remission following withdrawal of treatment. In a study of 36 patients with JIA who stopped anti-TNF therapy, an increased abundance of CD45RA−TNF+ memory CD4+ T cells deficient in immune checkpoint molecules (PD1−CD152−) prior to anti-TNF withdrawal was seen in patients who experienced flare compared with those who achieved sustained remission.[58] In this small study, the baseline ratio of CD45RA−TNFα+ to CD45RA+TNFα+ CD4+ T cells predicted flare with an AUC_{ROC} of 0.94. Whether this biomarker can predict drug-free remission remains to be established, as several patients in this study continued treatment with conventional synthetic DMARDs.

In another study of 47 patients with RA who stopped anti-TNF therapy (but continued methotrexate), lower frequencies of so-called inflammation-related CD4+ T cells ($CD4+CD45RB^{high}CD45RA^{+}CD45RO^{low}CD62L^{-}$)[59] were associated with sustained remission ($OR_{remission}$ 16.20, p=0.041).[60] However this and other flare-associated cellular subsets, such as the recently-discovered pre-inflammatory mesenchymal (PRIME) cells ($CD45^{-}CD31^{-}PDPN^{+}$),[61] remain to be studied in the context of drug-free remission.

Biomarkers of autoreactivity

As mentioned above, assessing the presence and diversity of autoantibodies provides an opportunity to define underlying antigen-specific mechanisms that can perturb immune homeostasis in immune-mediated inflammatory disease. Extending this concept, an increasing number of research groups are using modern and emerging laboratory techniques to quantify and monitor rare autoreactive cells in response to antigen-specific tolerogenic therapeutic strategies (discussed further in a previous review in this Series[62]). It is possible that cell-specific characteristics (e.g. surface markers, gene expression, cytokine production etc.) could be used as biomarkers of immune homeostasis. Such autoreactive cellular subsets could have either pro-inflammatory or pro-tolerogenic effects, and knowledge of their function could help illuminate underlying disease mechanisms. However, such biomarkers of autoreactivity are yet to be studied in the context of drug-free remission in immune-mediated inflammatory disease, and their suitability for translation to a commercially viable assay remains to be established.

Tissue biomarkers

Although convenient to acquire, peripheral blood-borne biomarkers can only infer the underlying disease processes occurring at the site of disease. Tissue biopsies, although usually more difficult to obtain, arguably provide the most comprehensive insight into immunopathology. This approach has already been used to study operational tolerance in liver transplantation, and more recently it has been applied to studies of drug-free remission in immune-mediated inflammatory diseases.

Using single cell RNAseq and multiparameter flow cytometry to study synovial biopsies from patients with RA, Alivernini *et al*[63] identified a higher abundance of MerTK$^+$CD206$^+$ synovial tissue macrophages in patients in remission versus those with active disease. Furthermore, in patients in remission who tapered biologic therapy (but continued methotrexate), a low baseline proportion of MerTK$^+$CD206$^+$ synovial tissue macrophages was predictive of subsequent disease flare (OR 13.5, 95% CI 2.3 - 80.8). Intriguingly, *in vitro* studies have shown that MerTK$^+$ synovial tissue macrophages produce significantly lower concentrations of pro-inflammatory cytokines and more IL-10 in response to LPS stimulation compared with their MerTK-negative counterparts. This, coupled with MerTK-dependent upregulation of a distinct transcription factor signature in MerTK$^+$ synovial tissue macrophages from patients in remission (but not those with active RA or healthy controls), suggests a potential pro-tolerogenic role for this cellular subset in RA remission. Whether these cells are predictive of drug-free remission in RA remains to be confirmed.

In a study of Crohn's disease, 45 patients in clinical and endoscopic remission discontinued infliximab (but continued maintenance conventional synthetic DMARDs) and were followed for up to 104 months.[64] Normalised mucosal expression of *TNF* in baseline colonic biopsies was predictive of sustained remission, with a median relapse free survival of 20 in those with normal *TNF* expression compared with 5 months in those with high expression. Again, whether these results are applicable to the context of drug-free remission has not been examined.

Microbiome

Recent years have witnessed a surge of interest in commensal human microbiota and their modulation of host immunity. The intimate relationship between microbiome and host suggests a wide range of potential mechanisms for the promotion of immune-mediated inflammatory diseases, including formation of neoautoantigens by post-translational modification of host antigens, molecular mimicry, mucosal permeabilisation, and polarisation of immune responses.[65] Strong associations have been observed between the presence of specific species of commensal bacteria and both the onset and severity of a range of immune-mediated inflammatory diseases. For example, an increased abundance of the intestinal commensal bacterium *Prevotella copri* is observed in individuals with early RA versus healthy controls,[66] with anti-*P. copri* antibody titres correlating with serum T helper-17 cytokine profiles;[67] furthermore, transfer of *P. copri* derived from RA patients can induce arthritis in experimental murine models.[68]

In contrast, it is likely that specific commensal bacteria may instead exert anti-inflammatory effects to maintain immune tolerance. For example, several studies in patients with IBD have observed lower levels of commensal organisms that produce and convert butyrate in patients versus healthy controls;[69] butyrate has been shown to exert anti-inflammatory and intestinal homeostatic roles in both animal and human studies.[70]

Therapeutic manipulation of the microbiome in order to create a more favourable balance of anti-inflammatory versus pro-inflammatory microbes is being actively investigated across a range of immune-mediated inflammatory diseases. Microbiome manipulation may be achieved through the use of dietary modification, probiotics, prebiotics (i.e. agents that promote the expansion of pro-tolerogenic microbes), and most strikingly through the use of faecal transplantation from healthy donors.[71] In a recent meta-analysis of four studies including 277 patients with ulcerative colitis, faecal transplant recipients were twice as likely to achieve remission after 8 weeks compared to placebo recipients (RR 2.03, 95 % CI, 1.07 to 3.86).[72] With these emerging results, it appears feasible that microbiome-related biomarkers could provide a possible measure of immune homeostasis. At present no published studies have explored the potential association between microbiome biomarkers with drug-free remission in immune-mediated inflammatory disease, although we expect this to be an area of development in the near future.

Limitations of current studies

Recent studies have offered tantalising glimpses of potential biomarkers of immune homeostasis in immune-mediated inflammatory diseases, which have shown impressive prognostic performance in small clinical cohorts. Nevertheless, there are several limitations common to these studies. First and foremost is a lack of validation of biomarkers in large independent cohorts; to date, no biomarker of immune homeostasis in these diseases has been externally validated. However, this remains an active area of research with several tolerance biomarker validation studies currently in recruitment: for example, the ongoing BIOlogical Factors that Limit sustAined Remission in rhEumatoid arthritis (BIO-FLARE) study [ISRCTN16371380]).

Furthermore, there remains a lack of standardisation across drug cessation studies, including the definition of remission used and differences between study populations in terms of demographics (e.g. age), disease stage (i.e. early vs. established disease), and previous drug treatment (e.g. previous biologics vs. biologic-naïve). Study quality also varies, particularly in terms of power, with overfitting of complex datasets a common limitation. The net effect of these non-standardised protocols is to make it very difficult, if not impossible, to compare and analyse results across different studies. In addition, the laboratory biomarkers explored by different studies are largely non-overlapping, with the exception of routine clinical serology such as ACPA and RF in RA.

Finally, there is a lack of consensus on the optimal approach to drug cessation, including the timing (e.g. abrupt vs. tapering) and the order of drug withdrawal in those treated with combinations of therapies. Indeed, very few studies have explored the comparative effectiveness of different strategies for drug cessation. In the TApering strategies in Rheumatoid Arthritis (TARA) study, 189 patients in remission were randomised to taper and then stop conventional synthetic DMARDs prior to anti-TNF, or vice versa, with a non-significant trend of increased drug-free remission in those who tapered conventional synthetic DMARDs first (20% versus 11%; p = 0.07).[73] Whether such an effect would reach statistical significance in a larger study remains to be seen and, furthermore, would need to be balanced against the relative prescription costs and side effect profiles of each individual therapy.

Translation to clinical practice

Several hurdles remain in translating the use of biomarkers of immune homeostasis to routine clinical practice (**Figure 2**). Assuming that immune homeostasis biomarkers are successfully validated (an essential first step), it will then be necessary to demonstrate their clinical efficacy in biomarker-driven protocols of drug withdrawal. Trials comparing no stratification (i.e. drug cessation in all) versus biomarker stratification (i.e. drug cessation only in biomarker positive group) are required to demonstrate superior ratios of remission to flare for biomarker-driven strategies, and thus provide evidence of clinical efficacy and, ultimately, cost-effectiveness of this approach. For example, the ongoing Liver Immunosuppression Free Trial (LIFT - NCT02498977) uses such a design to assess the efficacy of a biomarker-driven strategy of immunosuppression withdrawal in the setting of liver transplantation.

Following proof of efficacy, it would then be necessary to develop robust assays suitable for clinical use. Such assays would need to be compliant with necessary good clinical practice standards, yield accurate and reproducible results, and ideally be compatible with existing equipment and staff skillsets within healthcare clinical laboratory facilities. Furthermore, these assays would have to be acceptable to patients, and ideally be minimally invasive to avoid risk of significant adverse effects (especially when applied to clinically well sustained remission populations). Finally, the assays would need to be commercially viable by identifying a sufficient proportion of patients who are able to achieve drug-free remission, such that the treatment cost savings to healthcare providers outweigh the manufacturing, marketing, and sample processing costs of the assays.

Immune tolerance - a future target of disease control?

Assuming biomarkers are successfully validated and provided their efficacy is confirmed and commercially viable clinical assays are developed, what impact can we expect to see on clinical practice?

One major impact would be to facilitate the widespread adoption of personalised drug tapering and cessation in the routine clinical management of patients with immune-mediated inflammatory diseases. Current techniques to measure disease activity, while successfully used to identify those in need of escalation of therapy in treat-to-target approaches, are typically poor at discriminating those at the opposite end of the spectrum who can achieve drug-free remission. In this context, effective biomarkers of immune homeostasis would enable a personalised prediction of drug-free remission, identifying those most likely to benefit from drug withdrawal.[35] This would minimise the risk of disease flare, thus reducing potential flare-related complications, limiting use of resources needed to regain disease remission, and increasing patient acceptability. Furthermore, longitudinal monitoring of such biomarkers following drug cessation might identify those in whom immune homeostasis is subsequently perturbed, allowing for drug holidays followed by the pre-emptive re-initiation of therapy before disease flare occurs (**Figure 3**).

One further likely impact would be to provide insights into mechanisms that maintain immune homeostasis, or tolerance, in immune-mediated inflammatory disease. A greater knowledge of such mechanisms may identify potential therapeutic targets by which immune tolerance can be restored,

catalysing a novel class of tolerising therapies in immune-mediated inflammatory disease. Tolerance biomarkers should also provide the tools to identify those patients most likely to benefit from such therapies, and to quantify their clinical response.

Conclusion

A growing body of research has provided evidence for a range of potential biomarkers of drug-free remission across different immune-mediated inflammatory diseases. Such biomarkers of immune homeostasis have the potential to usher in a new era of disease management, in which the success of current treat-to-target approaches in the early stages of disease control are later augmented by personalised tapering and cessation of immunomodulatory treatment. Combined with the potential for tolerising therapies, it is possible that the current gold-standard treatment target of remission could be replaced in the future by drug-free remission, at least in some patients.[74] Considerable research, innovation, and commercial development will be required to achieve this. However, reflecting on the remarkable progress in immune-mediated inflammatory disease management over the past three decades, and recent progress in identifying potential biomarkers of immune homeostasis, another therapeutic revolution in immune-mediated inflammatory disease management may be closer than we think.

Search strategy and selection criteria

We identified references by searching PubMed with combinations of the terms "biomarker", "drug free remission", "tapering", "withdrawal", "cessation", "autoimmune", "operational tolerance" and various IMIDs including "rheumatoid arthritis", "psoriatic arthritis", "ankylosing spondylitis", "systemic lupus erythematosus", "inflammatory bowel disease", "psoriasis", "uveitis", "multiple sclerosis", "nephrotic syndrome", "vasculitis", and "juvenile idiopathic arthritis". We only reviewed English-language articles published in peer-reviewed journals, and deliberately focussed on reports of human disease rather than animal studies. The final reference list is not an exhaustive systematic literature review, but rather a selection of highlighted articles applicable to our aim of presenting a broad overview of current research in biomarkers of immune homeostasis in human IMID.

Competing interests

KFB reports grants to explore drug-free remission in rheumatoid arthritis from the British Society for Rheumatology, Academy of Medical Sciences, JGW Patterson Foundation, and Wellcome Trust. KFB and JDI are named inventors on a patent application by Newcastle University ("Prediction of Drug-Free Remission in Rheumatoid Arthritis"; International Patent Application Number PCT/GB2019/050902), and have an ongoing collaboration with Genentech to measure cytokines/chemokines in rheumatoid arthritis. JPXS declares no competing interests.

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Table - Summary of interventional studies of drug withdrawal that have identified biomarkers of drug-free remission in IMID.

Author	Disease	Number of patients	Treatment	Duration of follow-up	Proportion maintaining remission	Biomarkers	Effect size
Baker *et al*. (2019)[14]	Rheumatoid arthritis	44	csDMARDs	6 months after DMARD withdrawal	21/44 (48%)	Composite score of 3 $CD4^+$ T cell transcripts + IL27 + Boolean remission	ROC_{AUC} flare(95% CI) = 0.96 (0.91–1.00)
El Miedany *et al*. (2016)[24]	Rheumatoid arthritis	157	Arm 1: Half dose bDMARDs Arm 2: half dose bDMARDs and csDMARDs Arm 3: stop bDMARDs, half dose csDMARDs Arm 4: stop all DMARDs Arm 5: continue all DMARDs	12 months after DMARD withdrawal	Arm 1: 18/31 (58%) Arm 2: 13/32 (41%) Arm 3: 10/31 (32%) Arm 4: 7/31 (23%) Arm 5: 30/32 (94%)	Positive ACPA	OR_{flare} Arm 1 = 5.35, $p < 0.02$ Arm 2 = 5.68, $p < 0.01$ Arm 3 = 5.46, $p < 0.01$ Arm 4 = 8.64, $p < 0.003$

Author	Disease	Number of patients	Treatment	Duration of follow-up	Proportion maintaining remission	Biomarkers	Effect size
Figueiredo *et al*. (2017)[32] RETRO study	Rheumatoid arthritis	94	Various biological and non-biological DMARDs, prednisolone≤5mg/day	12 months after enrolment	Not stated	AMPA reactivity	Proportion flare: 0-1 AMPAs = 18% 2-5 AMPAs = 34% >5 AMPAs = 55% X^2 = 6.46, p = 0.011
Gerss *et al*. (2012)[48]	Juvenile idiopathic arthritis	188	MTX	At least 12 months after MTX withdrawal	120/188 (64%)	Elevated calprotectin (≥690ng/ml) Elevated S100A12 (≥175ng/ml)	HR_{flare} (95% CI) Calprotectin = 2.24 (1.39 – 3.62, p = 0.0009) S100A12 = 2.81 (1.70 – 4.65, p<0.0001)
Haschka *et al*. (2016)[15] RETRO study	Rheumatoid arthritis	101	Various biological and non-biological DMARDs, prednisolone ≤ 5mg/day	12 months after enrolment	13/27 (48%) withdrawal arm (versus 22/36 (61%) reduction arm and 36/38 (84%) continuation arm)	Positive ACPA	OR_{flare} (95% CI) = 5.23 (1.10 – 24.87), p = 0.038

Author	Disease	Number of patients	Treatment	Duration of follow-up	Proportion maintaining remission	Biomarkers	Effect size
Klarenbeek *et al*. (2011)[16] BeSt study	Rheumatoid arthritis	508	Various DMARDs	5 years	115/508 (23%) achieved DMARD-free remission, of which 59/115 (51%) maintained remission at 5 year time-point	Positive ACPA Positive RF	OR_{flare} (95% CI): Positive ACPA = 5.3 (2.4–11.8), $p < 0.05$ Positive RF = 2.9 (1.3–6.4), $p < 0.05$
Nishimoto *et al.* (2014)[40] DREAM study	Rheumatoid arthritis	187	TOC +/- glucocorticoids	52 weeks after TOC cessation	17/187 (9.1%)	Low serum IL-6 (< 35 pg/ml) Normalised MMP-3	HR_{flare} (95% CI): IL-6 = 0.41 (0.27–0.63) MMP-3 = 0.29 (0.19–0.43)
Rech *et al*. (2016)[46] RETRO study	Rheumatoid arthritis	94	Various biological and non-biological DMARDs, prednisolone $\leq$ 5mg/day	12 months after enrolment	63/94 (67%)	Moderate/high MBDA score	OR_{flare} 8.5 ($p = 0.004$)

Author	Disease	Number of patients	Treatment	Duration of follow-up	Proportion maintaining remission	Biomarkers	Effect size
Teitsma *et al.* (2018)[54] U-Act-Early study	Rheumatoid arthritis	60	MTX, TOC, HCQ, TNFα inhibitor	2 years after enrolment	37/60 (62%)	Elevated CCL18 Elevated CCL20 Elevated sIL2-Rα (TOC + MTX group)	Negative effect estimates (β): CCL18 = -3.31, p = 0.047 CCL20 = -1.24, p = 0.035 sIL2-Rα = 1.40, p = 0.039
Teitsma *et al.* (2018)[56] U-Act-Early study	Rheumatoid arthritis	60	MTX, TOC, HCQ, TNFα inhibitor	2 years after enrolment	37/60 (62%)	Histidine metabolism (TOC + MTX) Arachidonic acid metabolism (TOC) Arginine and proline metabolism (MTX)	Histidine metabolism, p < 0.001 Arachidonic acid metabolism, p = 0.018 Arginine and proline metabolism, p = 0.022

ACPA = anti-citrullinated peptide antibodies, AMPA = anti-modified protein antibodies, bDMARDs = biological disease-modifying anti-rheumatic drugs, CCL18 = C-C chemokine ligand 18, CCL20 = C-C chemokine ligand 20, CCRP-2 = anti-cyclic citrullinated peptide 2, CDAI = clinical disease activity index, CI = confidence interval, csDMARDs = conventional synthetic disease-modifying anti-rheumatic drugs, DAS28-CRP = disease activity score 28 joint count with C-reactive protein, DAS28-ESR = disease activity score 28 joint count with erythrocyte sedimentation rate, DFR = drug-free remission, DMARDs = disease-modifying anti-rheumatic drugs, HCQ = hydroxychloroquine, HR = hazard ratio, IL-6 = interleukin 6, MBDA = multi biomarker disease activity, MMP-3 = matrix metalloproteinase 3, MTX = methotrexate, na = not applicable, OR = odds ratio, RF = rheumatoid factor, ROC_{AUC} = area under the receiver operating characteristic curve, RR = relative risk, SDAI = simplified disease activity index, sIL-Rα = soluble interleukin 2 receptor alpha, SJC28 = swollen joint count in 28 joints, STM = synovial tissue macrophages, TJC28 = tender joint count in 28 joints, TNFα = tumour necrosis factor alpha, TOC = tocilizumab, ULN = upper limit of normal.

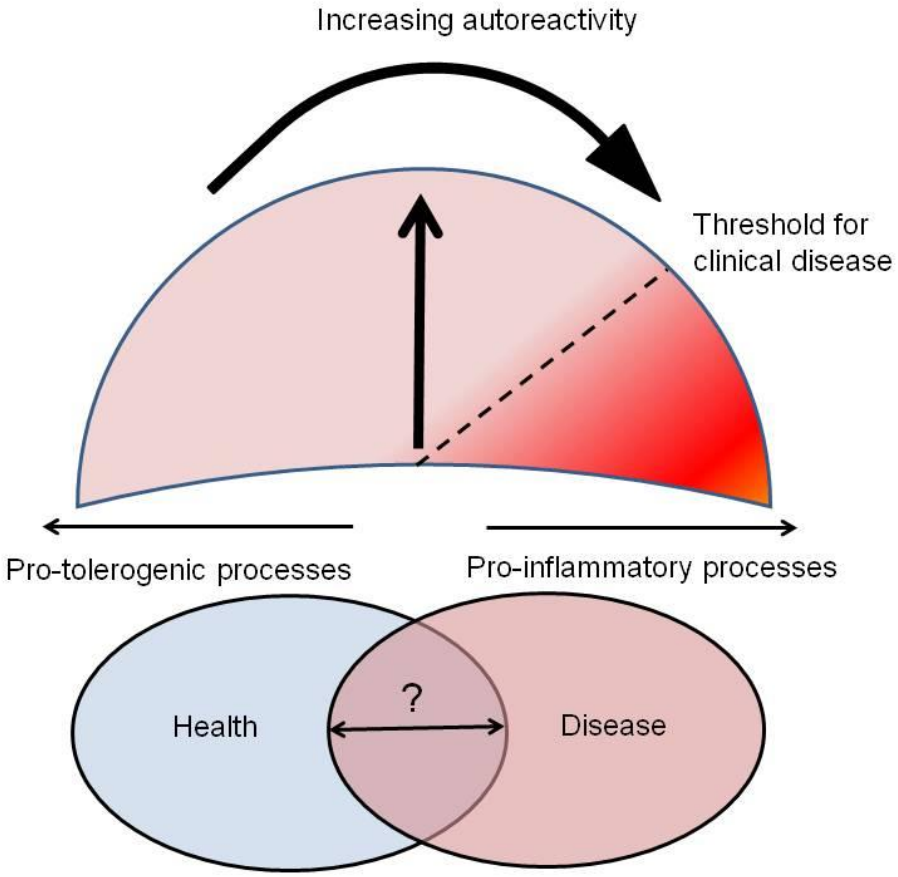

Figure 1 - Theoretical model of immune homeostasis as a balance of pro-inflammatory and pro-tolerogenic mechanisms, with clinical disease manifesting above a threshold of autoreactivity. Pro-inflammatory processes dominate in disease states and vice versa in health. The extent to which these mechanisms overlap, and whether specific compensatory pro-tolerogenic mechanisms exist exclusively in health or disease, remains uncertain.

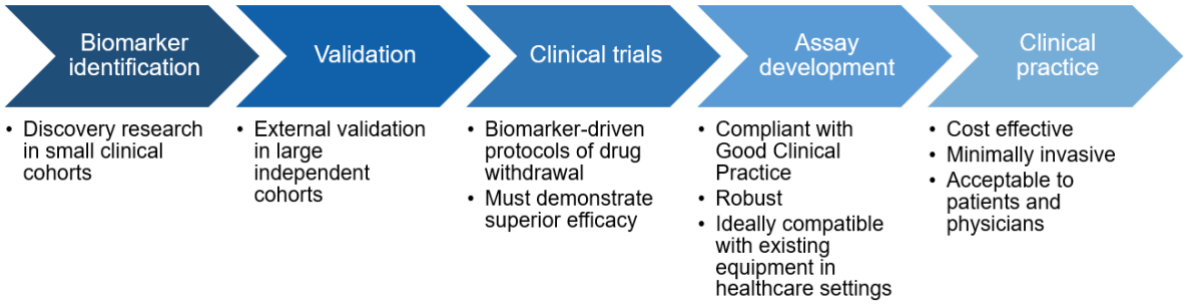

Figure 2 - The necessary steps in translation of biomarkers of drug-free remission from discovery research through to clinical practice.

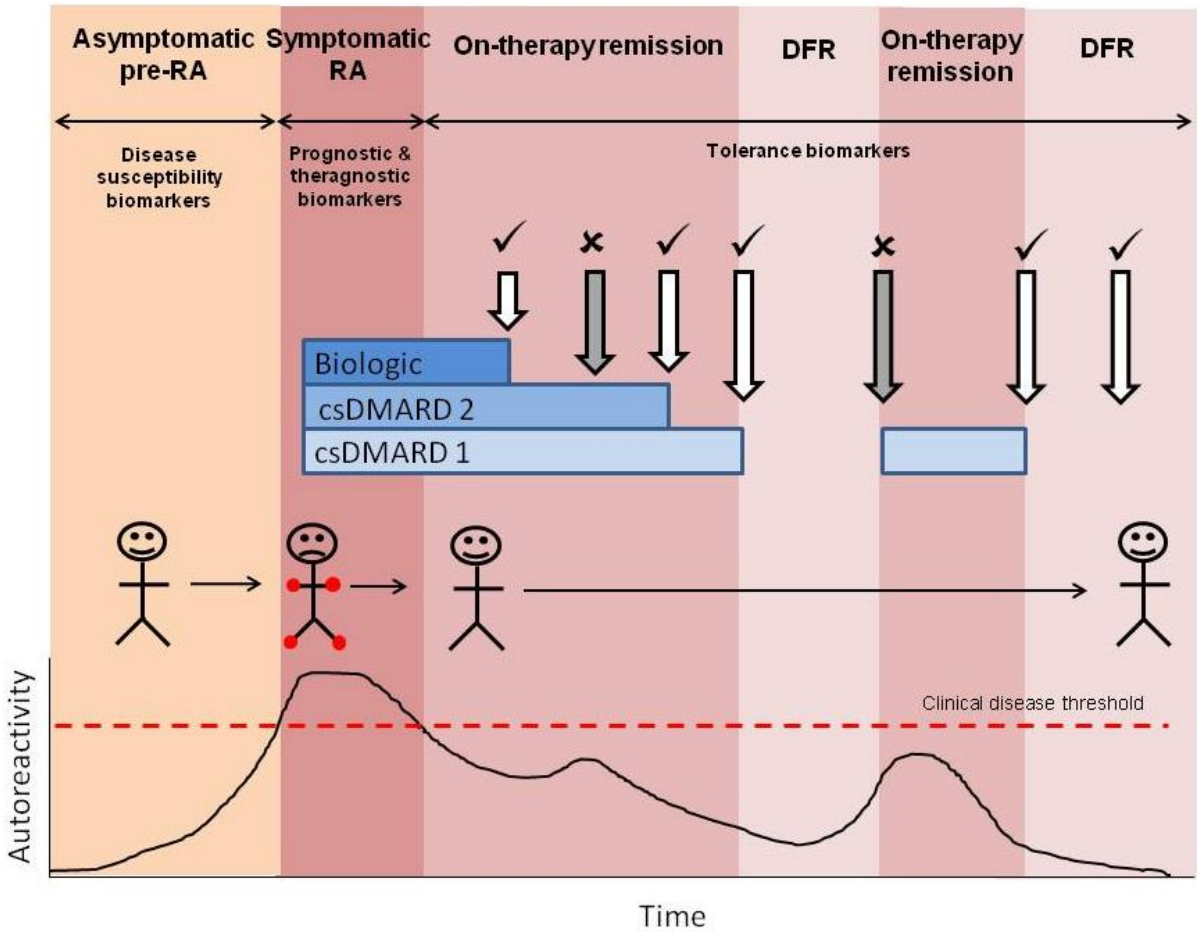

Figure 3 - Schematic representation of the potential application of future biomarkers to a personalisation of therapy in rheumatoid arthritis. Biomarkers of disease susceptibility could be used in the asymptomatic pre-RA stage, to guide use of novel strategies to prevent clinical disease. Once clinical disease is manifest, prognostic biomarkers would allow identification of those patients destined to develop severe disease, allowing early instigation of combination therapy with choice of agent guided by theragnostic biomarkers. Once disease remission is achieved, longitudinal measurement of immune homeostasis biomarkers would permit a personalised withdrawal of immunomodulation. Permissive biomarker test results (white arrows) would identify when drug tapering is appropriate, or when drug-free remission (DFR) can be extended where this has already been achieved. In contrast, non-permissive results (grey arrows) would indicate when drug regimens should be continued or reinstated, preventing relapse of clinical disease.