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Antigen-specific therapy of rheumatoid arthritis

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Background: Immunotherapy offers the promise of antigen-specific suppression of pathological immune responses in conditions such as autoimmunity and organ transplantation. Substantial advances have been made in recent years in terms of understanding basic immunological mechanisms of autoreactivity, as well as clinically implementing immune-based therapies that are antigen nonspecific. *Objective*: To provide an integrated overview of the current state of the art in terms of antigen-specific tolerance induction, as well as to predict future directions for the field. *Methods*: Examples of successes and failures of antigen-specific immunotherapy were sought. Particular attention was paid to the well-established collagen II-induced model of arthritis. *Results/conclusions*: Previous failures of antigen-specific immunotherapy were associated with lack of identification of clinically relevant antigens, as well as inappropriate tolerogenic methodologies. The advances in proteomics combined with novel gene-specific immune modulatory techniques place today's translational researchers in a unique position to tackle the problem of antigen-specific immunotherapeutic protocols.

Keywords: autoimmunity, dendritic cells, gene silencing, immune modulation, immunotherapy, rheumatoid arthritis, siRNA

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1. Introduction

Rheumatoid arthritis (RA) is characterised by inflammatory reactions in the synovial membranes and articular structures of joints. Approximately 1% of the general population is believed to be affected with peak incidence of onset during the 4th and 5th decades of life. RA is diagnosed based on various schemes, and one prevalently used scheme developed by the American Rheumatology Association requires the patient to meet 4 out of 7 of the following qualifying criteria: i) morning stiffness lasting longer than 1 h before improvement; ii) soft tissue swelling (arthritis) involving 3 or more joint areas; iii) arthritis of the hand; iv) bilateral involvement of targeting joints (e.g., symmetric proximal interphalangeal and metacarpophalangeal joints); v) positive serum rheumatoid factor (RF); vi) rheumatoid nodules; and vii) radiographic evidence of RA [1]. The conventional wisdom has traditionally been to initiate treatment with NSAIDS, and if response is not achieved, the patient is treated with more potent approaches such as disease-modifying antirheumatic drugs (DMARDS), which include methotrexate, sulfasalazine, leflunomide, hydroxychloroquine, minocycline and ciclosporin. Gold salts, D-penicillamine, and azathioprine were historically considered DMARDS but are rarely used today as front line therapy. In addition, controversy exists regarding whether corticosteroids should be classified as DMARDS. Within the class of DMARDs are new biological therapies such as abatacept (CTLA4-Ig),

anakinra (IL-1 receptor antagonist), infliximab (chimeric anti-TNF-α antibody), adalimumab (fully human anti-TNF-α antibody), and etanercept (soluble TNF-receptor). Although some recommendations have advocated aggressive front line therapy with DMARDs in order to inhibit joint damage early in disease progression [2], there is some debate as to whether enough data supports the use of biologicals for this purpose.

To date, treatment interventions have all been associated with non antigen-specific inhibition of inflammatory processes. For example, administration of infliximab together with methotrexate was demonstrated to be superior to methotrexate alone, according to the clinical American College of Rheumatology response criteria in Phase III double-blind trials [3]. However, the systemic suppression of TNF-α associated with infliximab use has caused concerns regarding increased susceptibility to infections. For example, in a retrospective analysis of 709 patients treated with at least one TNF-α blocker, 34.5% of patients reported infectious complications during the treatment period, with 6.2% falling under the category of serious infections. This incidence was statistically significant in comparison to control patients not having undergone TNF-α-blockade [4]. Particularly of concern is reactivation of latent infections such as tuberculosis, which was reviewed by Wallis [5]. In addition to the possibility of infectious complications, nonspecific immune modulation caused by systemic inhibition of TNF-α has been demonstrated in some situations to augment generation of antinuclear antibodies, although clinical implications of this are unclear at present [6,7].

Despite the fact that TNF-α blocking strategies substantially reduce signs and symptoms of RA, complete resolution of disease is hardly ever obtained. A meta-analysis of 26 efficacy and 18 safety clinical trials using biologicals for treatment of RA revealed no significant difference in efficacy between anti-TNF-α antibodies (infliximab) and soluble TNF-α receptor (etanercept). However, a non-significant trend showed decreased efficacy of recombinant IL-1 receptor antagonist (anakinra) [8]. Given that clinical inhibition of inflammatory cytokines does not lead to a long-term cure, and that various adverse effects are associated with these approaches, a more promising approach would be the induction of antigen-specific tolerance towards autoantigens causative of RA.

2. Collagen II as an autoantigen

Design of antigen-specific immunotherapies requires identification of self molecules that are not only associated with autoimmunity but are causative. For example, given the ability of the immune system to selectively evolve during the course of an immune response, antigens found to be recognised by the time the disease is in its late phases may not necessarily be the antigens that originally initiated autoimmunity. One manifestation of this phenomenon is the process of epitope spreading [9]. Numerous antigenic targets have been identified in RA patients including: human cartilage glycoprotein 39 [10], citrullinated protein and peptides [11], and various members of the heat-shock protein family [12,13]. For the purposes of this review the authors focus their discussion on one particular autoantigen, collagen II, which has served as one of the main autoantigens in animal models of RA [14-16]. Although there is some data supporting the clinical relevance of collagen II as an autoantigen [17,18], not all RA patients express autoreactivity to it [19], and in fact one report suggests it is not essential in disease progression [20]. Nevertheless, due to the well-described biochemical and immunological characteristics of collagen II, the authors use this as a model for our discussion, taking into consideration that collagen II-based immunotherapy may have clinical pitfalls. However, the authors believe that this discussion can serve as a framework for other recently described autoantigens that are surfacing as a result of the application of genomic and proteomic techniques to the study of autoimmunity.

Under non-inflammatory conditions, collagen II is sequestered from the immune system, serving as a fundamental component of hyaline cartilage. It is believed that inflammation, either mechanical or mediated by other means, causes release of this antigen and subsequent immune recognition. Some studies have demonstrated that various biochemical modifications of collagen II are associated with antigenicity and propensity for autoimmunity [21,22]. One of the important aspects of collagen II as an autoantigen is that an RA-like disease can be induced in various animal models by immunisation. For example, it was discovered in 1977 that rats immunised with collagen II in the presence of Freund's adjuvant developed a chronic proliferative synovitis resembling human RA [23]. This 'collagen-induced arthritis' (CIA) model was subsequently adapted to mice and rabbits and used in the evaluation of hundreds of experimental treatments for RA [24]. Importantly, many of the drugs used clinically for the treatment of arthritis have first been demonstrated as efficacious in the CIA model. These include infliximab [25], etanercept [26] and anakinra [27]. The CIA model is also an accepted model for the preclinical evaluation of potential RA therapeutics. Given that collagen II appears as a candidate for one of the major autoantigens in RA, as well as the fact that induction of immunity to collagen II is associated with disease onset, the authors focus their discussion on previous approaches that have sought to inhibit collagen II-specific immune responses.

3. Oral tolerance

One of the oldest known methods of antigen specifically modulating immune responses is the oral administration of the antigen. The process of oral tolerance is known to act not only through direct neutralisation of antigen-specific T cells but also through the induction of T-cell subsets,

which suppress antigen-reactive T cells [28]. Due to specialised antigen-presenting cell function in the intestine, it has been demonstrated using numerous antigens that TGF-β-secreting T_H3 cells may be induced subsequently to antigen ingestion [29-32]. Other antigen-specific cell populations that inhibit immunity after oral tolerance induction include $CD4^+CD25^+$ T regulatory cells [33,34] and IL-10 secreting Tr-1 cells [35]. In addition to generation of antigen-specific inhibitory cells, the process of oral tolerance is also believed to induce direct anergy of effector cells through several mechanisms, one of which being activation of T-cell receptors (TCRs) in the absence of a second signal, leading to abortive activation and default tolerance induction [36]. Therefore, oral tolerance induction is associated with at least two general mechanisms of immune regulation, the first being generation of regulatory T cells that actively suppress antigen-specific immune responses, whereas the second mechanism is neutralisation of existing antigen-specific cells through the process of anergy induction.

The ease of oral tolerance induction, at least the conceptual ease, has attracted numerous investigators to use this method to treat the CIA model. In 1986, Nagler-Anderson *et al.* [37] demonstrated that intragastric administration of collagen II was capable of reducing the incidence of CIA in mice. Protective effects of oral tolerance induction with collagen II were also seen in the adjuvant arthritis rat model, implying importance of this antigen in a variety of arthritis subtypes [38]. Mechanisms of oral tolerance induction in the CIA model were demonstrated by subsequent investigators to include reduction in IgG2b-collagen II specific antibodies, as well as generation of a T-cell population that was capable of transferring tolerance to naive mice [39]. The problem with these experiments is that the tolerogenic protocol was performed before disease onset, which is not clinically relevant. When oral tolerisation is attempted using conventional feeding after disease induction, no protective or therapeutic effect is seen [40]. However, investigators have demonstrated that various manipulations of the administrative method can potentiate tolerogenic effects. For example, Kim *et al.* [41] used a biodegradable polymer called poly(lactic-co-glycolic acid) to prepare nanoencapsulated collagen II. Such alternative delivery techniques are designed to allow for greater retention of antigen in the Peyer's patches and to conceptually induce tolerance with greater potency. Indeed, the investigators were able to demonstrate not only prophylactic but also therapeutic (e.g., post-disease onset) protection from CIA. Tolerance was associated with reduction of IgG2b, inhibition of antigen-specific T-cell proliferative responses, and generation of TGF-β expressing T cells.

Due to its apparent simplicity, the oral tolerance induction approach has been attempted clinically in numerous autoimmune diseases. For example, in Crohn's disease, where autoantigens are not as well defined as in other autoimmune diseases such as RA, Margalit *et al.* [42] used the novel approach of feeding autologous colonic extracts under the belief that the antigenic mixture would contain numerous entities including those responsible for stimulating auto-reactivity. A total of 31 patients with moderate-to-severe Crohn's disease were enrolled in a double-blind, placebo-controlled study in which the treated group received biopsy-extracted purified proteins in an oral formulation that the investigators termed Alequel. In terms of clinical responses, the inflammatory bowel disease questionnaire score improved in 43% of the patients receiving treatment versus 12% in placebo controls. Immunologically, a decreased number of T cells producing IFN-γ in response to autologous extract was observed in patients who achieved treatment-induced remission. Despite these results, the study was criticised due to the clinical end points chosen [43]. Overall, there appears to be lack of evidence supporting the superiority of oral tolerance induction in comparison to other commonly used approaches in the treatment of Crohn's disease [44]. Nevertheless, these clinical studies have demonstrated 'proof of concept' that antigen-specific immune modulation is clinically feasible. Other proof of concept studies supporting antigen-specific immune modulation in patients have been performed in multiple sclerosis patients [29,45,46], as well as systemic sclerosis [47] and Hashimoto's thyroiditis [48]. Unfortunately, despite the 'hint' of tolerogenicity, clinical improvements with oral tolerance induction are repeatedly minor, or not comparable to standard clinical treatments. For example, in RA, a 92-patient double-blind study involving switching from methotrexate to administration of oral collagen II demonstrated statistically significant deterioration of patient condition in the group that was switched to oral collagen II [49]. This and numerous other studies [50-53] suggest that at present oral tolerance induction is not an ideal clinical treatment of autoimmunity. Therefore, novel methods are needed for potentiating tolerogenic effects of oral tolerance before this procedure can become a clinical reality.

4. Immunisation with regulatory epitopes

The biochemical breakthroughs that have allowed for sequencing of MHC I and II bound peptides have led to the discovery that different epitopes derived from the same protein may induce tolerance or immunogenicity. For example, in the non-obese diabetic (NOD) model of spontaneous diabetes, the protein glutamic acid decarboxylase (GAD) is known to be one of the major autoantigens. Immunising NOD mice with the GAD 524-543 peptide protects from diabetes whereas the GAD peptide 534-553 offers no protection [54]. Protection from disease mediated by different peptide epitopes of the same protein is believed to be associated with deviation of cytokine secretion from inflammatory to anti-inflammatory, although the mechanistic basis for this is still under debate. Another main diabetes autoantigen, proinsulin, also displays similar characteristics in that immunisation with insulin B chain peptide (p9-23) endows protection from diabetes, whereas other epitopes of

insulin are non-protective or accelerate onset [55]. In the case of RA, specific immune inhibitory epitopes of the autoantigen heat shock protein (hsp) 65, as well as collagen II, have been used to induce protective immunity [56].

The clinical use of epitopes from autoantigens is potentially dangerous due to the possibility of stimulating or exacerbating autoimmune responses. The immunological phenomenon of epitope spreading has been documented in numerous systems and causes some degree of concern in situations where vaccination is used as a monotherapy for autoimmunity [9,57-59]. Despite this, numerous clinical trials with either autoantigenic epitopes in native form, or altered peptide ligands generated to specifically induce inhibitor responses, have been performed. One company (BioMS) is in Phase III trials for treatment of multiple sclerosis by vaccination with the amino acid residues 82 – 98 of human myelin basic protein. Phase II studies have demonstrated that administration of this peptide results in statistically significant improvements in subsets of patients possessing human leukocyte antigen haplotypes DR2 and/or DR4 [60]. Other groups have also initiated clinical trials using various epitopes of myelin basic protein in multiple sclerosis [61], although to date, clinical responses have not been superior to standard antigen-nonspecific approaches [62]. In RA, although immune suppressive epitopes of collagen II have been identified in animal models that induce protection from disease [63], the majority of clinical work has been performed targeting the autoantigen hsp 60 [64,65]. For example, a clinical trial using hsp 60 peptides demonstrated clinical remission in patients with juvenile RA [66]. As in the aforementioned examples of oral tolerance induction, clinical responses associated with peptide immunisation have not yet offered significant benefits in comparison to antigen-nonspecific therapy.

5. Augmentation of tolerance induction: understanding the processes

From the above discussions it becomes clear that: i) antigen-specific immunotherapy is feasible; and ii) in its present state the antigen-specific suppression is too weak for widespread clinical implementation. In order to develop more potent protocols, it is important to overview the mechanisms associated with antigen-specific tolerance. In the broadest sense, induction of tolerance can be associated with either directly inhibiting the effector T cells, or inducing cell populations that are capable of generating other cells with antigens specifically inhibiting the effector T cells.

During T-cell activation, the naive T-cell requires three signals: i) an antigen-specific signal that triggers the TCR; ii) a membrane-bound costimulatory signal; and iii) soluble signals that also serve costimulatory functions. Tolerance-inducing peptides or altered peptide ligands are believed to mediate suppressive effects, in part by causing a suboptimal activation of the TCR (e.g., lack of ZAP-70 requirement) [67]. Such partial activation causes not only functional anergy but sometimes results in deviation of the cytokine production profile [68]. Indication of oral tolerance, on the other hand, is associated with activation of T cells by specialised antigen-presenting dendritic cells with tolerogenic properties [69]. Specifically, antigen presentation in the gut is associated with lack of costimulatory signals [70], or upregulated production of 'co-inhibitory' signals such as programmed death-1 ligand [71]. The fact that oral tolerance is associated with specific antigen-presenting cells is supported by studies demonstrating cross presentation of ingested antigens by specific dendritic cell subtypes, as well as direct induction of tolerance/cytokine deviation [72].

Amplification of tolerogenic responses may be achieved by increasing the number of tolerogenic dendritic cells *in vivo* during times of oral tolerance induction. The cytokine flt-3 ligand has previously been demonstrated to act as a dendritic cell growth factor [73]. Accordingly, *in vivo* expansion of dendritic cells by systemic administration of flt-3 ligand has been demonstrated to potentiate tolerogenic responses after oral immunisation [74]. This approach, although attractive, possesses the possibility of concurrently increasing immune responses as flt-3L induces not only tolerogenic but also immune stimulatory dendritic cells [75]. Given the central role of the dendritic cell in stimulating naive T-cell responses and also generating T cells with regulatory properties, means of manipulating dendritic cells towards a tolerogenic phenotype appears to be a promising method of generating antigen-specific 'tolerogenic vaccines'.

6. Dendritic cells for antigen-specific tolerance

Previously it was demonstrated that the dendritic cell (DC) is the only antigen-presenting cell capable of activating naive T cells [76]. This is due to the high concentration of MHC II, as well as secondary signals and cytokine production by DC, in comparison to other antigen-presenting cells. The ability of these cells to act as 'cellular adjuvants' has made them a unique platform for the induction of antigen-specific immune responses. Numerous techniques and protocols have been generated that allow for the rapid expansion of autologous DC, pulsing of DC with antigens of interest, and readministration of DC for clinical immune response induction. The first DC vaccine to be commercialised involves autologous monocyte-derived DCs pulsed with biopsy-derived antigens called DCVax-Brain by the company Northwest Biotherapeutics. In addition, Dendreon has completed Phase III clinical trials using autologous dendritic cells pulsed with prostate-specific protein antigens [77] and is anticipating FDA approval in late 2007. In addition to immune stimulatory abilities, DCs are unique in their ability to stimulate antigen-specific T regulatory cells. The authors have previously reported that immature DCs possessing tolerogenic properties (Tol-DC) are found in transplant-tolerant recipients and are capable of generating alloantigen-specific

$CD4^+CD25^+$ T regulatory cells [78]. Subsequent to the authors' studies, it was determined that Tol-DCs possess numerous characteristics that allow them to stimulate T-cell differentiation into T regulatory cells. These characteristics include: i) expression of TGF-β [79]; ii) expression of IL-10 [80]; iii) upregulated expression of programmed death-1 ligand [81]; and iv) lack of costimulatory molecules and stimulatory cytokines [82]. As Tol-DC isolated from tolerant animals are not clinically relevant, methods are needed to generate *in vitro* cell populations that possess such tolerogenic characteristics. These methods include: i) generation of immature DC; ii) transfection of DC with agents that inhibit the immune response; and iii) blocking stimulatory signals on DC so that partial T-cell activation ensues. These methods will be discussed below.

7. Short-interfering RNA-based immunotherapy and vaccination

It was demonstrated more than a decade ago that culture of bone marrow in GM-CSF alone gives rise to a population of cells that resembles DC but lacks significant costimulatory molecule expression. When these cells were transferred into allogeneic recipients they were capable of prolonging cardiac transplant survival in a donor-specific fashion [83]. Other means of generating immature DC include the administration of agents that block critical intracellular transduction pathways associated with DC maturation. The authors' group has demonstrated that culturing DC in the presence of the IKK inhibitor LF (LF15-0195) gave rise to a similar immature DC population that was capable of prolonging allograft survival [84,85]. One of the reasons that immature DC induce tolerance and T regulatory cell generation is because of MHC II expression (signal 1) in the context of lack of costimulation (low expression of costimulatory molecules). According to this concept, the authors sought to generate Tol-DC through selectively silencing T_H1 stimulatory signals. As the cytokine IL-12 was previously demonstrated to play a critical role in the induction of T_H1 immunity by DC, the authors initially sought to block production of this cytokine through the use of short-interfering RNA (siRNA). Indeed, DC in which the IL-12 p35 subunit was silenced secreted higher levels of IL-10 in comparison with DC receiving mismatched siRNA. More interestingly, the silenced DC when pulsed with antigen could elicit a T_H2 recall response antigen specifically [86]. The attractiveness of using siRNA to modulate DC is that not only costimulatory signals can be modified but also general transcription factors or components of transcription factors. The authors have demonstrated that silencing of the NF-κB component RelB can not only induce generation of Tol-DC but that these Tol-DC are tolerogenic both in terms of blocking keyhole limpet hemocyanin (KLH)-specific responses, as well as blocking alloreactivity in a cardiac model of transplantation [87]. Given that silencing of immune stimulatory genes in DC can be used to tailor-make Tol-DC with specific tolerogenic properties, the authors have initiated preclinical studies to develop tolerogenic vaccines that are antigen specific using siRNA silenced DC. In the authors' proof-of-principle study they demonstrated that administration of collagen II pulsed DC in which IL-12p35 was silenced, could not only induce prophylactic protection but also inhibited disease progression after immunisation with collagen II and adjuvant [88].

8. Expert opinion

Antigen-specific immune modulation is one of the 'holy grails' of immunology. The realisation of this goal would not only offer a cure of serious autoimmune diseases but would also permit organ transplantation in absence of continual immune suppression. Clinically the DC has been validated as a 'platform' cell that can induce effective antigen-specific immune stimulation. Accordingly, there is a great drive to also use DC for induction of antigen-specific tolerance. Chemical and biological means of generating immature DC with Tol-DC properties have been previously used, however this approach is difficult to translate clinically due to lack of control over the cell phenotype generated. For example, growth of DC in low GM-CSF cultures results in Tol-DC with inhibited costimulatory molecules but also inhibited expression of MHC II [83]. As MHC II is involved in activation of T regulatory cells, it may be more beneficial to 'engineer' Tol-DC that express diminished levels of costimulatory molecules but basal levels of MHC II. Indeed, this is the advantage of siRNA technology as applied to generation of Tol-DC.

Gene silencing by siRNA allows for the development of numerous types of Tol-DC that can be tailor-made to address unique immunological needs. For example, by transfecting DC with gene-specific siRNA, the investigator may choose to generate cells that lack a specific cytokine, a costimulatory molecule, or a combination thereof. Furthermore, through silencing of upstream transcription factors that control general properties of DC, Tol-DC can be generated that are deficient in a plethora of T-cell activator functions. From a translational research perspective, siRNA silencing possesses one major advantage to traditional gene therapy approaches: the fact that genes are taken away and not added. Regulatory agencies have numerous concerns about administration of cells containing exogenous genes or endogenous genes that are overexpressed. These concerns range from uncontrolled expression causing long-term adverse effects including cancer transformation or horizontal gene transfer [89].

At present, experiments in our laboratory are combining the use of siRNA-generated Tol-DC with other tolerogenic protocols in order to synergise effects. For example, oral tolerance induction gives rise to $CD4^+CD25^+$ Treg cells that are antigen-specific but the levels of these are short-lived. One possibility under investigation in our laboratory is the use of 'booster' Tol-DC vaccines so as to maintain/amplify

levels of antigen-specific T regulatory cells in the periphery after initial oral induction.

Although clinically used agents in RA such as infliximab act in an antigen-nonspecific manner, there are numerous reports that infliximab increases the number of Treg cells [90-92]. This may be because the temporary inhibition of inflammatory reactions allow for a 'holiday' in which natural self-regulatory processes may start mediating protective effects. Other antigen-nonspecific immune modulators that temporarily block autoimmunity have also been demonstrated to be associated with transient rise of Treg cells [93]. Accordingly, the use of Tol-DC vaccines will more than likely be introduced initially to synergise with existing therapeutics. One of the immediate challenges will be the characterisation of key autoantigens in RA. The recent advances in immune recognition of citrullinated self-proteins offer support that new insights may be close at hand in this regard. Given that the clinical entry and successes of DC immunotherapy is not a reality, we believe the road has been paved for the similar use of DC immunotherapy for tolerance induction in treatment of autoimmunity in an antigen-specific manner.

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Declaration of interest

T Ichim is Chief Scientific Officer of Medistem Laboratories. The company operates in the area of stem cells and therefore there is no conflict of interest with this paper.

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