Heat Shock Proteins as Target for the Induction of Antigen-Specific Tolerance in Rheumatoid Arthritis and other Chronic Inflammatory Diseases

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Abstract: The critical causative factor of chronic inflammatory diseases such as rheumatoid arthritis (RA) is the faulty regulation of self-tolerance. Despite good results in patients that do respond to potent immunosuppressive therapies, in most of the cases only partial responses are achieved leaving them unduly susceptible to risks, such as infections. More importantly, immunosuppressive measures do not alter the basic condition, so that disease returns when therapy is halted. Antigen-specific therapies may represent a better and more physiological approach for manipulating the immune response avoiding the generalized immune suppression in patients and possibly leading to a state of permanent disease remission. The selection of auto-antigens without necessarily being the initiator of disease and with the ability to induce regulatory T cells is crucial for the development of antigen-specific therapies. Heat Shock Proteins (HSPs) are up-regulated during inflammation and HSP-responses are immuno-dominant. HSP-derived peptides have proved to be able to produce a shift from a pro-inflammatory to a tolerogenic phenotype in pathogenic T cells and endogenous HSP have been shown to act as targets for anti-inflammatory Tregs that control disease without general immune suppression.

Keywords: Immunological tolerance, regulatory T cells, CD4+ T cells, antigen- specific therapies, rheumatoid arthritis.

1. INTRODUCTION

The development of autoimmune diseases reflects a loss of tolerance for self-antigens in the immune system. This process involves genetic predisposition and environmental factors that alter its fine balance towards autoreactivity [1]. The exact aetiology of these diseases remains to be fully elucidated. However, significant advances have been made in the study of the cellular mechanisms involved in pathogenesis, which is now accepted to be characterized by the concerted action of different cells. Although the relative importance of different cell subsets early in these diseases is still debated, there is ample evidence showing that antigen recognition by autoreactive T cells in genetically susceptible individuals with deficient regulatory mechanisms trigger a complex network of events that leads to tissue damage [1]. Several suppressive drugs target different steps in this process, but since they affect the normal function of the immune system there is a good reason for the development of new approaches. Antigen-specific therapies represent, at least in theory, a better way for regulating the immune system by avoiding the generalized immune suppression in patients. In this article we provide an overview of the mechanisms that control self-tolerance in the immune system with a special emphasis on regulatory T cells (Tregs). The role of CD4+ T cells and pro-inflammatory cytokines in the pathogenesis of chronic inflammatory diseases is presented considering the case of Rheumatoid arthritis (RA). Finally, we discuss the potentialities and pitfalls of antigen-specific therapies in the treatment of chronic inflammatory diseases and the rationale of heat shock proteins as targets for this approach.

2. MECHANISMS OF IMMUNE TOLERANCE

Immune tolerance comprises a range of physiological mechanisms (central and peripheral) by which the immune system ensures a non-responsiveness to self-components and avoids excessive responses to foreign antigens thereby limiting collateral tissue damage [2]. Since the breakdown of immune tolerance can lead to a variety of autoimmune or allergic diseases, there has been intense research into the mechanisms that control this process.

Central tolerance involves a complex process whereby antigen-specific T cells are eliminated in the thymus if they express high-affinity receptors for self-components. This process is dependent on the autoimmune regulator Aire, a transcription factor that promotes ectopic expression of tissue specific antigens

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on medullary thymic epithelial cells [3]. T cells that express low-affinity receptors for self-components escape negative selection, and join the mature T-cell repertoire. Some T cells with potentially autoreactive receptors also escape negative selection. In the periphery, autoreactive T-cell clones are kept in check by regulatory mechanisms which involve their attenuation or deletion and/or the expansion and activation of Tregs [4]. In particular, Tregs have received a considerable attention because they have shown potential in suppressing pathological immune responses in autoimmune diseases [1, 2].

2.1. Suppressive Mechanisms of Tregs

Two main subsets of Treg cells have been described depending on their origin: natural occurring or thymus-derived Treg cells and induced or peripherally-derived Treg cells. Recently, the use of thymus and peripherally-derived Tregs has been recommended because these terms offer information regarding the anatomical location of their differentiation [5]. Thymus-derived Tregs are generated as a consequence of high-avidity interactions (but below the threshold required to induce apoptosis) between their T-cell receptor (TCR) and major histocompatibility complex (MHC) molecules on the surface of stromal cells [6]. TCR stimulation with relatively higher intensities induces forkhead box protein P3 (FoxP3) expression, a transcription factor that plays an important role in Treg cell development and function [7]. However, its expression is not sufficient for conferring and maintaining the function and phenotype of Tregs. It has been proposed that Treg-cell-specific epigenetic changes are also critical in this process, which appears to depend on the duration of TCR stimulation [8]. TCR stimulation for an appropriate length of time produces the characteristic DNA hypomethylation pattern of the FoxP3 region in Tregs. A plausible model has been proposed where FoxP3+epigenome+ T cells are driven into a stable Treg cell lineage, whereas FoxP3+ epigenome- T cells are unstable and might lose FoxP3 expression [8].

FoxP3 has been considered the most reliable phenotypic marker of thymus-derived Tregs [7]. In addition, they constitutively express the high affinity IL-2Ra chain (CD25), cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4) and glucocorticoid-induced TNF receptor-related protein (GITR), among others. However, none of them seems to be an exclusive marker of Tregs since other subsets of T cells can express these molecules in some conditions [9]. Low level expression of CD127 (IL-7Rα) has been also proposed as a Treg marker which correlates with FoxP3 expression and their suppressive capacity [10]. In order to become suppressive, Tregs need to be first activated via TCR in an antigen-specific fashion. However, once activated, Tregs can also suppress immune responses of T cells with other specificities and antigen presenting cells (APC) in a process known as ‘bystander suppression’ [11]. Immunosuppressive effects of Tregs include both contact-dependent mechanisms through CTLA-4 signalling in T cells and APC as well as contact-independent mechanisms through secretion of regulatory cytokines such as interleukin (IL)-10, tumour growth factor beta (TGF-β) and IL-35. Preferential engagement of CTLA-4 with CD80/CD86, instead of CD28, provides a negative proliferative signal in effector T cells [12]. CTLA-4 expressed on Tregs may signal dendritic cells to produce high levels of the enzyme indoleamine 2,3-dioxygenase, which causes the degradation of tryptophan, an essential amino acid for T cell proliferation [13], and consequentially inhibits their proliferation. In addition, Tregs can also down-regulate the expression of co-stimulatory molecules on APC enabling the inhibition of antigen presentation to effector T cells thereby generating tolerance [14]. Direct killing of conventional T and B cells, monocytes, and dendritic cells by human Tregs has also been reported [15, 16]. IL-2 plays a critical role in survival and maintenance of thymus derived Tregs in the periphery. Although Tregs express constitutively the α chain of the IL-2 receptor, the synthesis of this cytokine is barely detectable in Tregs which therefore depend on exogenous IL-2 for their survival [7]. Based on this, it is proposed that IL-2 consumption by Tregs is one immunosuppressive mechanism of these cells through which they can deprive peripheral conventional T cells from this cytokine [17]. More recently, a non-cell-autonomous gene silencing mechanism as a potential mode of Treg-cell-mediated suppression has been described, *via* microRNAs (miRNAs)- containing exosomes. Tregs seem able to package and deliver different proteins and RNA species (including miRNA) to various immune cells, including Th1 cells, suppressing their proliferation and cytokine secretion [18].

It has been reported that thymus-derived Tregs can transfer suppressive properties to conventional T cells in co-culture by contact-independent mechanisms mediated by IL-10 and TGF-β. This process, which is known as infectious tolerance, leads to the generation

of peripherally-derived Tregs [19]. These cells could be also generated at peripheral compartments from conventional T cells under very specific conditions of antigen exposure. Just like their natural counterparts, peripherally-derived Tregs have immunosuppressive properties and fulfil their function through mechanisms involving the secretion of cytokines and cell-cell contact [20]. It is possible to discern the existence of two subpopulations of adaptive Tregs: Tr1 cells that mainly produce IL-10 and Th3 cells producing TGF-β. These cells are essential for maintaining homeostasis in the gastrointestinal tract [20].

3. PATHOGENESIS OF RHEUMATOID ARTHRITIS

In individuals genetically susceptible to autoimmunity, one or several regulatory mechanisms are defective, resulting in the expansion and migration of autoreactive T cells to their targeted tissue where uncontrolled inflammation leads to tissue damage. Among autoimmune diseases, RA is most prevalent, affecting 1% of the world population [21]. This is a chronic and destructive disease characterized by synovial hyperplasia and joint inflammation which lead to cartilage and bone destruction. However, systemic features, including cardiovascular, pulmonary, and skeletal disorders are commonly present [22].

3.1. Role of CD4+ T Cells

The central role of CD4 + T cells in RA pathogenesis comes from the demonstration that the strongest genetic risk for RA is conferred by the HLA locus [23]. RA-associated HLA-DR4/1 molecules which contain a common amino acid motif (QKRAA) termed shared epitope, confer disease susceptibility by presenting different antigenic peptides to CD4 + T cells [24]. The hypothesis argues that CD4+ T cells may be stimulated by an autoantigen (specific to the joints or ubiquitous), or by a highly conserved foreign protein cross-reacting with its human homolog, or by a neo-antigen expressed as a result of posttranslational events. A number of possible antigens have been identified, including collagen type II, human cartilage glycoprotein 39 (gp39), heat shock proteins (HSP), and citrullinated proteins [25]. Antigen-activated CD4+ T cells stimulate macrophages and synovial fibroblasts to produce IL-1, IL-6, tumour necrosis factor α (TNF-α) and matrix-degrading metalloproteinases, which are major players of joint destruction in RA [22, 26]. Activated CD4+ T cells also stimulate B cells to produce immunoglobulins, including rheumatoid factor and anti-citrullinated protein antibodies (ACPA). The role of ACPA in RA pathogenesis remains unclear but their presence is strongly associated with strong genetic risk factors for RA such as: *HLA-DRB1* shared epitope and *PTPN22* and with one of the main environmental factors: smoking [27]. These autoantibodies which are highly specific for RA serve as a powerful serologic marker for early diagnosis of RA, as a prognostic predictor of more severe joint destruction and poor response to therapy [28]. B cells can also act as APCs and secrete pro-inflammatory cytokines (including TNF-α) [29]. Although RA has been considered as a disease mediated by type 1 helper T cells (Th1) producing interferon gamma (IFN-γ), attention has increasingly focused on the role of type 17 helper T cells (Th17), a CD4+ T cell subset that produces IL-17, 21, 22 and TNF-α [30, 31]. Pro-inflammatory cytokines such as: IL-1, IL-6, IL-21 and IL-23 as well as tumour growth factor beta (TGF-β) produced by macrophages and dendritic cells support Th17 differentiation and suppress Treg differentiation [32]. Moreover, Tregs fail to control disease due to defective function which seems to be secondary to the inflammatory environment of the synovia [33, 34]. It has been shown that Tregs from RA patients are unable to inhibit the secretion of pro-inflammatory cytokines such as IFN-γ and TNF-α even though they are competent at suppressing the proliferation of autologous CD4+ CD25- T cells [35, 36]. Altogether, it seems that the predominance of pathogenic effector T cells in the presence of impaired T-cell regulatory mechanisms contribute to the chronicity of inflammation in RA.

3.2. Role of Synovial Cells and Pro-Inflammatory Cytokines in Cartilage Damage and Bone Destruction

RA synovial fibroblasts (also termed fibroblast-like synoviocytes or type B synoviocytes), together with synovial macrophages, are the two leading cell types in the intima layer of synovia that invades and degrades cartilage and bone. Synovial fibroblasts normally regulate the composition of the synovial fluid and the extracellular matrix assuring the integrity of diarthrodial joints. However, in RA these cells assume an aggressive phenotype characterized not only by high levels of inflammatory cytokines but also by the loss of contact inhibition like in tumours, the expression of chemokines and adhesion molecules becoming a prominent component of the destructive pannus [37]. It seems clear that RA synovial fibroblasts not only act as passive responders to the inflammatory process initiated by CD4+ T cells, but also contain intrinsic alterations that convert them into destructive cells that

play a leading role [37]. Pro-inflammatory cytokines produced by synovial fibroblasts, macrophages and immune cells also affect the normal behaviour of other synovial cells such as: osteoblast, osteoclast and chondrocytes. Osteoclasts are specialized cells arising from cells of monocyte-macrophage lineage that secrete proteinases and create a local acidic environment that mediates bone destruction [38]. On the other hand, osteoblasts arise from mesenchymal stem cells with the capacity to produce and mineralize bone matrix [39]. Interactions between receptor activator of the nuclear factor kappa B (RANK) and its ligand (RANKL) are essential in osteoclastogenesis. Osteoblast-derived RANKL plays important role in generating osteoclasts in physiological conditions but the pathogenic role in RA is played mainly by immune cells and synovial fibroblast-derived RANKL. IL-1, IL-6, IL-17 and TNF-α stimulate the expression of RANKL on synovial fibroblasts and immune cells, enhancing RANK signalling between those cells and osteoclasts, which promote the activation of pathogenic osteoclasts [38, 39]. These cytokines may also play an important role in suppressing the functional capacity of osteoblasts to produce bone and to repair erosions [38]. Thus, pro-inflammatory cytokines inhibit regeneration of bones mediated by osteoblasts but stimulate its degradation by osteoclasts contributing to the degeneration of bone tissue. On the other hand, under the influence of IL-1, IL-17 and reactive nitrogen intermediates, the cartilage is progressively deprived of chondrocytes by apoptosis which in physiological conditions regulate matrix formation and cleavage [22]. By inducing chemokine production, IL-17 indirectly attracts numerous effector T cells, B cells, monocytes, and neutrophils to the inflamed joint [40]. On the other hand, IL-6 drives local T and B cell activation and autoantibody production [41]. Thus, this cytokine may contribute to the induction and chronicity of the autoimmune process through B-cell modulation and Th17 differentiation. Furthermore, IL-6 mediates systemic effects that promote acute phase responses, anaemia and lipid-metabolism dysregulation, among others [22].

Cytokine inhibitors against TNF-α [42], IL-1 and IL-6, a B cell depleting agent and a co-stimulation blocker [43] have been approved by the FDA for the treatment of RA. Despite excellent results in the patient group that does respond to these treatments in most of the cases only partial responses are achieved and a continuous treatment is required [44]. None of these treatments restore immune tolerance to the extent of sustained remission after therapy withdrawal. Therefore, the next challenge is to maintain disease remission with a minimal-treatment regimen. The development of drugs with a safer profile aimed at restoring immune tolerance mechanisms using antigen-specific therapies is a current focus of research.

4. THE RATIONALE OF ANTIGEN- SPECIFIC THERAPIES FOR RESTORING IMMUNE TOLERANCE MECHANISMS

In theory, antigen-specific therapies are a better approach for manipulating the immune response avoiding the generalized immune suppression in patients and providing a long lasting effect. The overall goal of antigen- specific tolerance is to present known autoantigens to the immune system in a way that they can elicit a regulatory response. In practical terms, an antigen introduced by oral route or in a soluble form tends to diminish rather than potentiate immune responses to the antigen. In fact, the nasal or oral administration of a pathogenic self-antigen typically found at the site of inflammation leads to considerable reduction of the severity of symptoms in many experimental models of autoimmune diseases [45-48]. This phenomenon is mediated by both the neutralization of antigen-specific T cells and the induction of TGF-β-secreting Th3 cells or IL-10-secreting Tr1 cells [49-51]. The activation of Tregs through the mucosal route is related to the presence of specialized APCs with tolerogenic properties in the gastrointestinal tract [52]. The mucosally induced antigen-specific Tregs are thought to migrate to the site of inflammation as their cognate antigen is expressed there. Apart from a proper routing of administration, another aspect to consider is that peptides should be administrated at the lowest dosage still producing a significant biological effect, so as to facilitate the expansion of specific T cells with a regulatory phenotype while minimizing the chances of cross-activate pathogenic T-cell clones. However, the optimal dosage has been difficult to translate from animal models to humans.

Antigen-specific Tregs have demonstrated higher potential than polyclonal expanded Tregs to treat autoimmune diseases [53]. One of the best examples is in the non-obese diabetic (NOD) mouse model where Tregs expressing a TCR specific for an islet antigen (BDC2.5) were more effective compared to polyclonal Tregs in controlling diabetes [54, 55]. However, one of the main questions is how to exploit a single antigenic specificity for control of autoimmunity

which involves many distinct self-antigens. Results from animal models have shown that induction of tolerance to a single self-antigen can efficiently prevent a polyclonal T-cell response due to mechanisms of bystander suppression. Bystander suppression was demonstrated in the previous mentioned work when BDC2.5 Tregs were able of controlling disease induced by splenic polyclonal T cells transferred into diabetic mice. The fact that the suppressive function of antigen-specific Tregs is not restricted to a single antigenic specificity raises hope concerning the efficacy of this alternative in clinical settings. In addition, the induction of new suppressor cells by infectious tolerance could be important for achieving long term suppression, which makes antigen-specific Tregs excellent targets for therapy in autoimmune diseases.

Immune tolerization trials in RA were initially focused on presumed triggers of the disease, such as peptides derived from collagen and gp39 [56, 57]. However, several trials using these antigens administered by the oral route in patients with RA and juvenile idiopathic arthritis (JIA) were unable to sort a clinical effect, although the treatments were well tolerated and safe [58, 59]. Likewise, the administration of glutamic acid decarboxylase or insulin, the major targets of immune adaptive response in type 1 diabetes, are effectively treating diabetes in NOD mice but so far failed to prevent or reverse the disease in humans [1]. These results led to rethink the use of presumed inducer self-antigens as potential therapeutic targets. Indeed, in humans when the autoimmune disease becomes clinically evident, the response has expanded beyond the original inducers, affecting additional self-antigens in a process known as 'epitope spreading' [60]. Through this mechanism, the disorder may fall into a self-perpetuating cycle where the identity of the original trigger could be irrelevant for clinical practice. Therefore, antigen-specific therapies should be focused on antigens playing a role in disease perpetuation/modulation. It should be emphasized that effective immune tolerization requires the induction of Tregs capable of bystander suppression. Thus, such antigens need to bind to disease-associated HLA molecules and should activate antigen-specific Tregs in order to be able to suppress pathogenic responses to their cognate self-antigen and other relevant antigens present at the site of inflammation. Ideally, antigens are needed to be up-regulated in the inflammatory process itself and in this way, once tolerance has been established, Tregs will not be activated thereby reducing immune suppression when not required. In general, antigen-specific therapies should be administrated to patients where the onset of autoimmune disorder is still recent, in an attempt to intervene before the reactivity to self-antigens had become widespread.

4.1. Heat Shock Proteins as Targets for Antigen-Specific Therapies

Heat Shock Proteins (HSPs) could be a source of those antigens. HSPs are intracellular molecular chaperones which are important for cell survival under stressful conditions [61]. The family consists of several families of molecules (HSP10, 40, 60, 70, 90, and 100) known for their strong evolutionary conservation, which results in a high level of homology between bacterial and mammalian HSPs. Under inflammatory conditions they are up-regulated in response to several mediators, like reactive oxygen species (ROS), TNF-α, IL-1 and 6. In fact, HSPs are abundantly present in synovial fluid and tissue of patients with RA, JIA or other arthritic autoimmune diseases [62-64]. Due to the high homology among species, microbial HSP-specific responses could cross-react with self-HSP which in theory could increase the risk for autoimmunity. However, in various cases, such responses were seen to correlate with a disease remitting course of events [65, 66]. In this way, HSPs may well be involved with a protective regulation of the inflammation. In fact, HSP-specific Tregs are involved in the regulatory response in self-limiting conditions; however, this mechanism is impaired in autoimmunity [67]. Thus, peptides aimed at restoring HSP-specific Tregs responses could result beneficially in dampening inflammation.

A protective role in experimental arthritis models has been demonstrated for numerous members of the HSP family. Self-cross-reactive responses have been reported to be important for the protective effect of peptides in this setting [62]. A simplified schematic representation of how priming with microbial HSP peptide may lead to induction of a self-HSP cross-reactive and protective T cell response is given in Figure **1**.

A conserved mycobacterial HSP70-epitope called B29 has its mammalian homologs abundantly present in murine and human MHC class II. The administration of B29 elicits potent primary antigen-specific Tregs that suppress disease in a mouse model of RA [68]. Moreover, the Treg population specific for B29 was long lived *in vivo* and its presence suppressed established disease in mice. These results suggest that

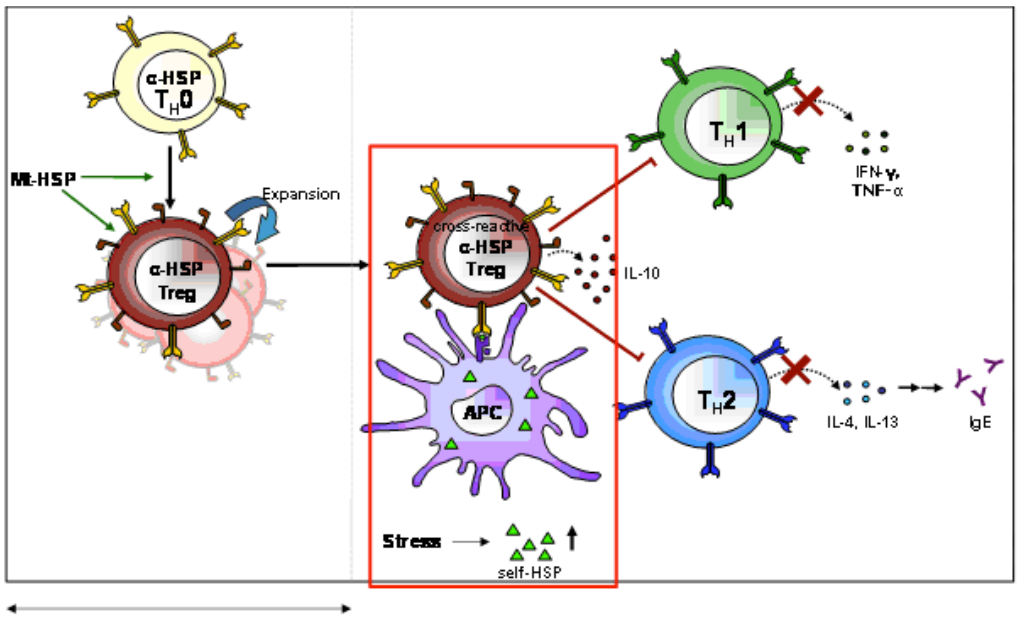

Figure 1: A *Mycobacterium tuberculosis* HSP (Mt-HSP) specific T cell response is induced by immunization with mycobacteria or Mt-HSP peptides. The resulting T cell responses include Treg. Upon cell stress in the tissues (such as in synovial tissues during the inflammation of the synovium during arthritis) the mammalian homologs of the conserved microbial HSP sequences are preferentially up-loaded in MHCII molecules of cells in the inflamed tissues. Upon cross-recognition of the self-homologs, Treg will produce anti-inflammatory cytokines such as IL-10, which down-regulate the production of mediators by both Th1 (inflammatory) and Th2 (allergy promoting) cells. Within the red square the crucial cross-recognition of self-HSP by the microbial HSP specific Treg is depicted.

HSP70-specific Tregs can have a great potential for antigen-specific immune interventions.

A similar mode of action may have been present in the case of dnaJP1, a peptide derived from bacterial HSP40 administered by the oral route in RA patients [69]. This peptide is similar to the corresponding peptide of its human homologue and contains the QKRAA motif associated with RA. A pilot Phase I trial with this peptide, recruiting 15 RA patients for a 6-month long treatment, found that the peptide was able to shift the pro-inflammatory phenotype of peptide-specific T cells to a regulatory phenotype, increasing the production of anti-inflammatory cytokines such as IL-4 and IL-10 while decreasing the levels of TNF-α and INF-γ [69]. The placebo-controlled pilot phase II trial involving 160 patients with active RA showed that the treatment was safe, well tolerated, and reduced the levels of TNF-α [70]. A progressive separation in clinical responses between the treatment and placebo group was achieved after an induction period which is consistent with an active tolerization process. In this study a positive clinical response was found to be associated with the expression of PD-1, B7-H1, B7-DC, CTLA-4 and FoxP3 prior to treatment initiation. These data suggested that pre-existent mechanisms associated with T cell tolerance and anergy may be needed to induce clinically effective tolerization.

DiaPep277, a 24-amino acid peptide derived from the 437–460 sequence of the human HSP60, was first discovered to arrest the progression of β-cell destruction in NOD mice [71]. Additional studies demonstrated that DiaPep277 also activated Tregs by interacting with their Toll-like receptor 2 [72]. The treatment of newly diagnosed diabetic patients with DiaPep277 was well tolerated, preserved the function of pancreatic β cells and decreased the demand for exogenous insulin when compared to the placebo groups in clinical trials phase I and II [73]. It seems that early treatment with DiaPep277® is promising in adults but it is not known whether continued treatment will have a beneficial effect preserving β-cell function in a long term. A clinical trial phase III was conducted to evaluate the safety and efficacy of DiaPep277 in preserving endogenous production of insulin in newly diagnosed type 1 diabetic adult patients [74, 75]. However, these papers were recently retracted due to a scientific misconduct in the analysis of the results. A confirmatory phase III trial is ongoing now in diabetic patients.

Results from clinical trials performed in patients with RA and type 1 diabetes mellitus show that HSP-derived peptides can be used to produce a phenotypic pro-inflammatory to a tolerogenic shift in pathogenic T-cell clones which could provide clinical benefits to patients

without the need for general immunosuppression. However, there is not conclusive data about the possibility of inducing long lasting regulatory effects. On the other hand, clinical efficacy has been less than expected and seems to be associated to some immunological/genetic factors yet not fully characterised. Thus, there is a real need of increasing the efficacy of antigen-specific therapies in clinical autoimmune diseases.

It has been proposed that the peptide used for an antigen- specific therapy should mimic the naturally processed epitope as close as possible, since altered peptide ligands (APLs) may behave unpredictably [76]. However, there are some situations in which the efficacy of soluble peptide therapy can be improved by making some changes in the peptide sequence.

4.2. Use of Altered Peptide Ligands in Preclinical and Clinical Settings

The induction of antigen-specific tolerance has been attempted by using antigenic peptides whose primary sequences have been altered to affect either their affinity to MHC class II molecules or to the TCR. There is ample published evidence on the capacity of APLs to modulate the immune response in experimental models of autoimmune disorders involving the induction of anergy or apoptosis of pathogenic T cells [77, 78] and bystander suppression through the induction of Tregs secreting suppressive cytokines [79, 80]. In general, APLs have outperformed their original peptides during testing in animal models. Efforts to dissect the mechanism of action of APLs have focused on the biochemical events leading to T-cell activation. Binding of the MHC-APL complex to the target TCR may produce altered phosphorylation patterns and modulate tyrosine kinase activity, thus generating changes in the response of these cells [81].

In particular, it has been documented that improving the affinity of a peptide for a MHC molecule creates a stronger tolerogen [76, 82]. For instance, a major encephalitogenic epitope of myelin basic protein (Ac1-9) is poor at inducing tolerance in experimental autoimmune encephalomyelitis, a model for multiple sclerosis, compared to peptides with increased affinities for class II molecules in which position 4 was changed [83]. Another example is that of the immuno-dominant epitope of human HSP60 (180-188), used by Prakken *et al.* to design an APL that produced, upon intranasal administration, a prophylactic and therapeutic effect vastly superior to that of the native peptide in a model of adjuvant-induced arthritis. This APL bound the MHC of rats (RT1B1) with higher affinity, inducing IL-10 Tr1 cells that controlled disease progression [84]. Domínguez *et al*. used bioinformatics to select new T cell epitopes from HSP60. The selected peptide was modified at position 4, a change that enabled its presentation in the context of several MHC class II molecules related to RA [85, 86]. HLA-DR restriction assays confirmed the nature of this peptide as being an HLA-DR-restricted CD4+ T-cell epitope [86]. Unlike its wild-type peptide, the APL was able to expand CD4+ T-cell clones with a regulatory phenotype in Balb/c mice and in peripheral blood mononuclear cells (PBMC) from RA patients and had a potent clinical and histopathological effect in one animal model of RA [85]. On the other hand, this APL induced apoptosis of activated CD4+T cells from PBMC of RA patients whereas naïve cells were not affected [86].

Despite excellent results in animal models, first clinical trials using two APLs derived from the immuno-dominant HLA-DR2-restricted T-cell epitope of myelin basic protein (residues 83-99) in patients with multiple sclerosis were halted due to safety concerns [87, 88]. Both studies were interrupted after the appearance of systemic hypersensitivity reactions and the worsening of disease symptoms in some patients associated to high doses of APL (50 mg) due to the expansion of T-cell clones with a Th1 phenotype. However, some improvement was observed in patients receiving the lower dose of APL (5 mg). As discussed before, peptides should be used at the minimal dosage still producing a biological effect in order to decrease the potential cross-activation of T-cell clones with a Th1 phenotype.

One of the few drugs successful in the clinic so far is Copaxone®, classified as an APL for its mechanism of action. This drug has been successfully used since the last decade for the treatment of multiple sclerosis. Copaxone® is a random sequence polymer synthesized from the aminoacids L-Ala, L-Tyr, L-Lys and L-Glu, which can be presented by numerous MHC class II molecules. This polymer acts as an antagonist of the immuno-dominant epitope of myelin basic protein provoking tolerogenic effects in autoreactive T cells [89].

In general, the excellent results obtained in animal models using antigen- specific therapies for restoring immune tolerance have been difficult to translate to humans. As antigen therapy in humans occurs in the

context of a more complex environment compared with laboratory animals, the achievement of remission will inevitably require the application of several complementing strategies. The previous or concomitant inclusion of antigen-specific therapies within established treatments using drugs or biologicals already approved is currently being examined with promising results.

5. COMBINATORY THERAPY

As discussed above, a pro-inflammatory environment is one of the hallmarks of active RA. Most studies indicate that Tregs in RA patients are not deficient, but rather that their functionality becomes compromised by this environment. Immunosuppressive therapies, such as TNF-α inhibitors or anti-CD3 therapy reduce signs and symptoms but also create an environment favouring the development of Tregs [36, 90]. Thus, the combination of immunosuppressive treatments with antigen-specific therapies could create a proper environment for the expansion of antigen-specific Tregs able to migrate to the target tissue.

Such was the rationale of a study combining the induction of antigen-specific Tregs with anti-TNF-α drugs in a rat model of adjuvant-induced arthritis. The nasal administration of the arthritogenic HSP60 peptide (p180-188) or a single dose of Etanercept® failed to produce a significant reduction of the clinical signs of the disease. However, a combined schedule consisting of the administration of a single dose of Etanercept® before the induction of mucosal tolerance with the HSP60 peptide, led to a significant decrease in clinical and histopathological scores [91]. A shift in the cytokine profile towards a regulatory phenotype was also observed in the combined schedule. Noteworthy, the results of the therapeutic combination were similar to those of a full three-dose course of Etanercept®, implying that one potential benefit of the combination could be a reduction in the number of doses of anti-cytokine therapy, together with its associated side effects.

Another example is the combined therapy of anti-CD3 with disease-related peptides which has been more effective than anti-CD3 or peptide alone in experimental models of new onset diabetes [92, 93].

There is also clinical evidence suggesting that efficacy improves when the induction of Tregs is combined with immunosuppressive treatments. In the clinical trial described earlier where the dnaJP1 peptide was administered to RA patients, the clinical effect was more noticeable in the group that also received hydroxychloroquine [70].

6. CONCLUSIONS

Significant advances have been made in the treatment of chronic inflammatory diseases using DMARDs and biologics. However, in most of the cases only partial responses are achieved, treatments require long term continuation causing immune suppression in patients that leads to complications. Peptide immunotherapy with bystander antigens such as HSPs shows promising results in experimental models through Treg induction, and results from clinical trials are currently emerging. However, there is not conclusive data about the possibility of inducing long lasting regulatory effects. Ongoing clinical trials exploring such effects on newly diabetic patients using DiaPep277 could help to get a more clear idea about this issue. However, as antigen therapy in humans occurs in the context of a more complex environment, it is likely that the treatment of autoimmune diseases will require a combinatorial therapy to restore long term tolerance and abrogate disease. Available data suggest that combining antigen-specific therapies with systemic treatments may provide considerable clinical benefits. Such combination could help to reduce the dose of anti-inflammatory treatments and therefore side effects as well as provide long lasting tolerogenic effects.

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DECLARATION OF INTEREST

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript.

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