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Targeting of tolerogenic dendritic cells towards heat shock proteins: a novel therapeutic strategy for autoimmune diseases?

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Keywords: tolerogenic dendritic cells, autoimmune diseases, heat shock proteins, regulatory T cells
List of abbreviations:

ACPAs: Anti-citrullinated peptide antibodies
APC: antigen presenting cell
DAMPS: danger-associated molecular patterns
ER: endoplasmatic reticulum
Hsp: heat shock proteins
IPEX: immunodysregulation polyendocrinopathy enteropathy X-linked syndrome
JIA: juvenile idiopathic arthritis
MITAP: minimal information model for tolAPC
NLRs: nucleotide-binding oligomerization domain (NOD)-like receptors
PAMPs: pathogen-associated molecular patterns
PRR: pattern recognition receptor
RA: rheumatoid arthritis
RLRs: retinoic-acid-inducible gene I like receptors
TLR: toll like receptor
ToIIC: tolerogenic dendritic cell
Tregs: regulatory T cell

Abstract
Tolerogenic dendritic cells (tolDCs) are a promising therapeutic tool to restore immune tolerance in autoimmune diseases. The rationale of using tolDCs is that they can specifically target the pathogenic T cell response, while leaving other, protective T cell responses intact. Several ways of generating therapeutic tolDCs have been described, but whether these tolDCs should be loaded with autoantigen(s), and if so, with which autoantigen(s), remains unclear. Autoimmune diseases, such as rheumatoid arthritis, are not commonly defined by a single, universal, autoantigen. A possible solution is to utilize surrogate autoantigens for loading of tolDCs. We propose that heat shock proteins (HSPs) may be a relevant surrogate antigen, as
they are evolutionary conserved between species, ubiquitously expressed in inflamed tissues and have been shown to induce regulatory T cells, ameliorating disease in various arthritis mouse models. In this review, we provide an overview on how immune tolerance may be restored by tolDCs, the problem of selecting relevant autoantigens for loading of tolDCs, and why HSPs could be used as surrogate autoantigens.

1. Restoring immune tolerance to ‘self’ in autoimmune disease: a promising clinical intervention

Immune tolerance is crucial for preventing destructive immune responses to self-tissues. In healthy individuals, immune tolerance is maintained at different levels: in the thymus, where T cells that strongly react to self-antigens are deleted; and in the periphery, where self-reactive T cells that escaped negative selection in the thymus are kept in check by regulatory cells. A breach in immune tolerance facilitates immune attacks on self-tissues that, when becoming dysregulated, lead to chronic autoimmune disorders.

Regulatory T cells (Tregs) play a pivotal role in maintaining immune tolerance in the periphery. They are a heterogeneous population of cells that can either be derived from the thymus (naturally occurring Tregs) or can be induced in the periphery from naïve CD4+ T cells (induced Tregs). They exert their suppressive action on immune effector cells through a number of distinct mechanisms, including inhibition of antigen-presenting cell function, killing of effector cells, secretion of immunosuppressive cytokines and compounds, and interference with metabolic pathways (reviewed in 1,2).

Tregs are critical to preventing autoimmune disease. A total loss of functional Tregs, as seen in IPEX (immunodysregulation polyendocrinopathy enteropathy X-linked syndrome) patients, leads to severe autoimmunity affecting multiple organs 3.
In specific autoimmune diseases, however, it is thought that a more subtle change in the function of Tregs is involved in the pathogenesis. For example, although type I diabetes patients have similar numbers of Tregs as healthy controls, their Tregs display reduced suppressive activity and defects in IL-2 signaling. In rheumatoid arthritis (RA) patients, Tregs have reduced ability to suppress inflammatory cytokine production. Furthermore, enhanced numbers of Tregs co-expressing IL-17 were found in both the peripheral blood and synovial fluid of RA patients, suggesting conversion of Tregs into inflammatory cytokine-producing effector cells.

Restoration of Treg function is emerging as a promising clinical intervention for autoimmune diseases. One way of achieving this is by replenishing the Treg pool in autoimmune patients with functional Tregs, either by treating patients with drugs that selectively expand Tregs in vivo, or by generating new Tregs ex vivo before injecting them into the patient (reviewed in 2,9). However, a downside of this approach is that expanding Tregs ‘randomly’ may give rise to general suppression of the immune response, thereby increasing the risk of infection, and perhaps even cancer. A preferred approach would be to direct the Treg response to defined and relevant antigens that are being expressed in the target tissue. This would not only limit off-target immunosuppression, but would most likely also increase the efficacy of the Treg therapy, as was indeed shown in mouse models. An outstanding issue is, however, how to achieve the expansion of antigen-specific Tregs, and how to choose the relevant antigen(s). Here, we propose to use tolDCs to induce Tregs against heat-shock proteins that are ubiquitously expressed in inflamed target tissues, as outlined below.

2. Tolerogenic dendritic cells as a therapeutic tool

DCs are a heterogeneous family of professional antigen presenting cells (APC) that can be classified on the basis of their ontogeny, surface marker expression profile and their anatomical location. DCs are as important for the induction
of effective immunity against invading pathogens as they are for the maintenance of immune tolerance. Primary immunodeficiency patients with mutations in GATA2 have defective DC function, resulting in enhanced susceptibility to infection and cancer, but also to autoimmune conditions, most likely due to a reduction in Tregs.\(^{13}\)

The role of DCs in instigating immunity versus tolerance is largely determined by their maturation status. Under steady-state conditions, tissue DCs are immature, expressing low levels of MHC-II and co-stimulatory molecules; their ‘default’ setting is to induce tolerance. These immature DCs can become immunogenic when they sense pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) via pattern recognition receptors (PRRs). These include toll like receptors (TLRs), retinoic-acid-inducible gene I (RIG-I)-like receptors (RLRs), and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). PRR-mediated signaling plays a central role in the maturation process that DC need to undergo to acquire potent T-cell stimulatory properties.\(^{14}\) Fully matured DCs express high levels of MHC-II, co-stimulatory markers (e.g. CD86) and pro-inflammatory cytokines (e.g. IL-12p70, IL-23, TNFα), all required for the efficient induction of T effector cell responses. Furthermore, during DC maturation the expression of chemokine receptors is modulated (e.g. CCR5 is downregulated and CCR7 is upregulated) enabling DC migration towards lymphoid tissues to present antigen to naive T cells. However, the outcome of maturation of DC is not always the generation of DC with immunogenic properties. Certain DAMPs and immune suppressive compounds have been shown to drive the maturation of DC with tolerogenic properties (tolDC)\(^{15-18}\). These tolDCs may be phenotypically mature (i.e. high levels of MHC II and co-stimulatory molecules), but may express co-inhibitory molecules (e.g. PD-L1, PD-L2, ILT3), lack expression of pro-inflammatory cytokines and instead produce immunosuppressive cytokines and compounds (e.g. IL-10, TGF-β, IDO). The maturation status of these DC has been referred to as ‘semi-mature’. Thus, there is plasticity with regard to the functional maturation of DC, and the environmental
cues that DC receive during the maturation process determines whether they become immunogenic or tolerogenic.

DCs are able to mediate tolerance via several mechanisms. They can induce iTregs through, for example, membrane-bound PD-L1, which blocks the Akt/mTOR pathway to preferentially stimulate naive T cells to become iTregs. Furthermore, PD-L1 and PD-L2 provide inhibitory signals to both CD8+ and CD4+ T cells which drives the T cell into a state of tolerance. Secreted compounds such as IL-10, IL-27, TGF-β, retinoic acid and IDO, can convert naïve T cells into iTregs. DC can also promote T cell tolerance through T cell killing, and the induction of T-cell hyporesponsiveness (anergy).

The importance of DC in maintaining immune tolerance has led to exploring the therapeutic use of DC. Various ways have been described to create DC with stable tolerogenic properties, called tolDCs. The tolerogenic properties of these in vitro generated tolDCs depend on the specific method used (reviewed in ). For example, tolDCs generated with the immunosuppressive agents dexamethasone and/or the active form of Vitamin D3 (1α,25-Dihydroxyvitamin D3) are characterized by a semi-mature phenotype, with high levels of MHC II, intermediate levels of costimulatory molecules, low levels of pro-inflammatory cytokines and high levels of immunosuppressive cytokines IL-10 and TGF-β. TolDCs can also be genetically engineered, for example through the transduction of immunosuppressive or pro-apoptotic molecules (e.g. IL-10, CTLA-4, FASL) or silencing of immunostimulatory molecules (e.g. CD80/CD86, IL-12) (Reviewed in ). These different types of tolDCs have been shown to reduce or prevent autoimmune diseases or transplant rejection in animal models, providing important proof of principle evidence that these cells can be applied therapeutically. Their therapeutic benefit is associated with a reduction of pro-inflammatory effector T cells and NK cells, and the induction of regulatory T-cells or IL-10-producing T cells.
Efforts have been made to translate these findings from animal studies to the clinical setting. Good Manufacturing Protocols to generate tolDCs from human donor cells have been developed, and methods to preserve the tolDCs and reduce the production costs are being explored. Since there are diverse methods of generating tolDCs and other types of tolerogenic APC (tolAPC), a minimal information model for tolAPC (MITAP) was generated. MITAP enables researchers to report their data in a standardized and more transparent manner, facilitating data comparison and interpretation, ultimately paving the way for the development of standardized protocols for the production of tolDCs and other tolAPC for therapeutic application. A number of tolDCs have been tested in phase I clinical trials, including for type I diabetes, Crohn’s disease and rheumatoid arthritis. Encouragingly, tolDCs therapy in all these studies was found to be feasible and safe, providing rationale to conduct further studies into their efficacy.

3. The problem of targeting autoantigen(s) – which ones?

One of the main advantages of tolDC therapy is the specific targeting of pathogenic immune responses. Many of the drugs that are currently used to treat autoimmune diseases are non-antigen specific, leading to general immunosuppression. With tolDCs autoreactive T cells can, theoretically, be exclusively targeted. But how to achieve this is still a debate. A number of studies have provided clear evidence that tolDCs need to be loaded with a disease-relevant antigen to exert their beneficial immune modulatory action. Loading of tolDCs with type II collagen was required, for example, for antigen-specific disease remission in the collagen-induced arthritis model. More recent research shows that this is also applicable in other autoimmune diseases. Furthermore, when comparing the therapeutic action of unloaded tolDCs and tolDCs loaded with a disease relevant peptide (MOG40-55) in the experimental autoimmune encephalomyelitis (EAE) model, Mansilla et al showed that
although the unloaded tolDCs inhibited disease symptoms, the MOG\textsubscript{40-55} loaded tolDCs diminished disease even more \textsuperscript{45}.

In contrast, other studies have shown that disease remission can be established when administering unloaded tolDCs \textsuperscript{46,47}. This may suggest that tolDCs are able to take up the relevant antigen \textit{in vivo}. It has been hypothesized that unloaded tolDCs induce T cell anergy rather than promoting Tregs. These anergic T cells might be capable of suppressing excessive Th17 and Th1 responses \textsuperscript{48}. Non-antigen-pulsed tolDCs might also induce regulatory populations that do not require an antigen. For instance, B cells can be converted into Bregs partly through the production of retinoic acid by the tolDC \textsuperscript{49}. However, if these non-antigen-pulsed tolDCs are able to take up antigen \textit{in vivo}, one has to consider the safety of these tolDCs, since it is possible that the non-antigen-pulsed tolDCs also take up other antigens that should not be targeted.

Nonetheless, if tolDCs need to be loaded with antigen(s) prior to infusion, a remaining problem is the question of which antigen to use, and in what form. In many autoimmune diseases, including RA, the knowledge about the relevant autoantigen(s) involved is insufficient. Moreover, even if some of the relevant autoantigens are known, as is the case for multiple sclerosis (MS), the problem of HLA diversity remains \textsuperscript{44}. Some peptides (e.g. proteolipid protein) that have been shown to be involved in the pathogenesis of MS are restricted to a specific HLA-class (e.g. HLA-DQB1*0602), making it more difficult to standardise the peptides used for all MS patients \textsuperscript{50}.

For RA, no universal autoantigen exists. Several candidate self-proteins have been described in relation to the pathogenesis of this disease. Epitopes from joint-derived antigens such as collagen type II (CII) and human cartilage-derived glycoprotein HCgp39 are presented by DCs and macrophages to T cells in inflamed joints of RA patients \textsuperscript{51}. Furthermore, the endoplasmic reticulum (ER) stress-associated protein GRP78/BiP is described as a potential autoantigen. The ER stress
response is increased in RA synovial tissue and fluid and the ER chaperone, GRP78, is important for synoviocyte proliferation and angiogenesis, which are substantial indicators of RA.

Posttranslational modifications may also be important in generating novel epitopes that trigger autoimmunity. Anti-citrullinated peptide antibodies (ACPAs) are found in sera of 70-80% of RA patients. Immunogenetic studies have shown that more than 90% of the RA patients share a HLA-II epitope in the DRB1 chain (HLA-DRB1 *0101, *0401, *0404). This so-called shared epitope (SE) is also associated with ACPAs; SE-positive patients are predisposed to having ACPAs. Feitsma et al identified two HLA-DRB1 restricted CD4+ T cell clones that recognized citrullinated vimentin and were also present in the inflamed joint of RA patients. This indicates that CD4+ T cells can respond to naturally processed epitopes from an autoantigen.

The finding that ACPAs were present in inflamed joints of patients but not in the joints of healthy individuals, together with the discovery that citrullinated autoantigen specific CD4+ T cells were only found in PBMCs from RA patients, suggests that both the ACPAs and these CD4+ T cells play a significant role in the pathogenesis of RA. Scally et al (and others) provide molecular evidence on how CD4+ T cells are able to recognize citrullinated antigens. They also showed that in the autoantigen recognizing CD4+ T cell population of HLA-DRB1*04:01 RA patients, the percentage of Tregs (both activated and resting) was reduced, whereas the populations of naïve and effector memory CD4+ T cells were increased compared to healthy subjects. This indicates that citrullinated peptides are plausible autoantigens in RA.

To test if citrullinated antigens are good candidates for an immunomodulatory therapy, a phase I clinical trial was performed. In this study autologous in vitro generated tolDCs were exposed to citrullinated autoantigenic epitopes and administered intradermally into patients. The trial showed that the DC vaccination was safe and indicated an anti-inflammatory effect after DC administration. However, using citrullinated peptides has the consequence that therapy is limited to patients.
with HLA-DRB1 (*0101, *0401, *0404) and it is unknown if the reactivity in these patients is similar. We took a different approach in our recent phase I safety trial in patients with rheumatoid and inflammatory arthritis. TolDCs were loaded with autologous synovial fluid; the rationale being that this fluid contains relevant joint-associated antigens. The downside of this approach is that it is not always possible to obtain sufficient synovial fluid from RA patients for tolDC loading. Furthermore, as the antigens are unknown, it is difficult to monitor changes in the antigen-specific T cell response after tolDC administration.

The use of surrogate autoantigens could be a preferred option for the loading of tolDCs. Possible candidates are heat shock proteins (HSPs). HSPs are typically intracellular proteins, with no peptide leader sequences that can target secretion. However, there is evidence that HSP can have access to the extracellular milieu, either by passive or active mechanisms. Both the endogenous upregulation of HSP with so-called HSP co-inducers and the exogenous administration of (recombinant) HSP have led to immunomodulatory effects in various models of experimental autoimmunity. Therefore, HSPs could be used as surrogate autoantigen not only for RA but also for other auto-immune diseases. This will be discussed in further detail in the next section.

### 4. HSPs as surrogate autoantigens for autoimmunity

The main function of HSPs is to support folding and transport of a large variety of (misfolded) proteins as intracellular molecular chaperones. Their expression can be significantly upregulated under conditions of stress like fever, viral infection, nutritional deficiency, cold and exposure to the pro-inflammatory cytokines IFN-γ and TNF-α. Generally, HSPs can be classified into different families based on their monomeric molecular weight (HSP10, HSP20-30, HSP40, HSP60, HSP70, HSP90 and HSP100 families). Some HSP family members (e.g. HSP60 and HSP70) are
highly conserved throughout evolution, resulting in immunological cross-recognition of certain mammalian and microbial HSP homologues.

Initial observations that ignited studies on the role of HSPs in autoimmunity were made in the mycobacteria-induced adjuvant arthritis model in rats. Generated mycobacteria-specific T cell lines were shown to have arthritogenic potential and it was later discovered that HSP60 was the antigen recognized by the mycobacteria-specific T cell lines. Further studies followed showing that synovial fluid cells and peripheral blood mononuclear cells of chronic inflammatory arthritis patients could also respond to mycobacterial HSP60. In contrast, HSP60 responses were absent in control subjects. Moreover, monoclonal antibodies recognizing mammalian HSP60 were produced and it was found that HSP60 was expressed in synovial membranes of patients with chronic arthritis. Similar results were found for the HSP family members HSP40 and HSP70. Synovial fluid and peripheral blood T cells of RA patients could recognize a bacterial variant of HSP40, but healthy subjects or disease controls could not. In addition, the human homologues of HSP40 and HSP70 were found to be overexpressed in the synovial lining of the joints of RA patients.

Interestingly, numerous experimental animal models and even a few clinical trials have shown that treatment with (myco)bacterial HSPs can induce HSP-specific anti-inflammatory T cell responses. Experimental autoimmune disease models in both rat and mouse showed significantly reduced arthritis severity after prophylactic immunization with mycobacterial HSP60 or HSP70. Although the exact mechanism for disease amelioration is still not completely understood, suppression of arthritis is likely induced by IL-10 producing Tregs. One possible explanation for the propagation and/or induction of a regulatory phenotype in HSP60/70-specific T cells lies in the high homology between the bacterial and mammalian variants of the HSP proteins. Even though HSPs are considered immunogenic - microbial HSP60, for example, has been known as the so-called ‘common antigen of gram
negatives’ already before its molecular definition\textsuperscript{79} - the highly conserved parts of the proteins could induce a tolerogenic response as these can be recognized as self-antigens by the body’s own immune system\textsuperscript{80}. Moreover, since bacterial HSPs are mostly encountered in the tolerising gut or lung mucosa, conserved and thus repeatedly encountered HSP antigens are more likely to obtain a regulatory phenotype. In addition to conservation and microbial-self cross-recognition, HSP70 family members are directly involved with antigen processing and consequently, HSP70 fragments were found to be one of the most frequent cytosolic MHCII natural ligand sources\textsuperscript{81-83}. Presentation of HSP70 peptides may therefore be part of the earlier mentioned default tolerant state of the immune system, where MHCII presented HSP peptides are part of a continuous and credible target for Tregs. It is, however, important to keep in mind that in a dysregulated immune system like seen in patients with autoimmune diseases, antigens that would normally induce an anti-inflammatory immune response, could now potentially induce a pro-inflammatory response.

Since the HSPs used for the previous described experiments are from bacterial origin and can potentially induce an unwanted anti-inflammatory response towards these bacteria, a safer form of the HSPs is needed. One way to accomplish this is to use bacterial HSP-derived peptides that show high homology with the mammalian variant. The high homology to the self-antigen will prevent unwanted responses towards the bacteria and at the same time ensure cross-reactivity with the mammalian HSPs presented in the inflamed joint. Indeed, two of the three clinical trials using HSPs as therapy were performed with HSP-derived peptides. A pilot phase II trial using a HSP40-derived peptide, dnaJP1; which also contains the ‘shared epitope’\textsuperscript{84}, was tested in juvenile idiopathic arthritis (JIA) patients. After oral administration of the dnaJP1, a change from a pro-inflammatory to a tolerogenic T cell response to dnaJP1 could be observed \textsuperscript{85,86}. In a second phase II trial, an HSP60-derived peptide, DiaPep277, was used to treat patients with type I diabetes. It
was found that DiaPep277 was safe and showed a trend towards a greater preservation of beta-cell function as compared to controls. In a third recent trial, a mammalian HSP70 family member, BiP, was tested in RA patients. In this case, whole protein was administered intravenously. The results of this phase I/II safety trial showed no serious adverse drug reactions. Moreover, at the higher treatment doses disease remissions were seen in some cases.

As discussed earlier, one potential disadvantage of using peptides is HLA diversity in patients. Consequently, HSP peptides need to either 1) be able to bind multiple HLA-DR molecules, including the RA associated HLA-DRB1 *0101, *0401, *0404 molecules, or 2) a peptide pool of several HSP peptides able to bind one or more of the RA associated HLA-DR molecules needs to be administered. For HSP60 and HSP70 several pan-DR peptides have been discovered. Kamphuis et al used a computer algorithm to identify both self and bacterial HSP60 peptides able to bind a number of distinct HLA-DR haplotypes. They found several peptides that were able to bind the major RA/JIA-associated HLA-DR molecules and T cells from both JIA and RA patients were able to respond to five out of eight peptides. In addition, de Wolf et al showed that an HSP70 peptide, B29, also binds multiple HLA-DR molecules. They concluded that more than 80% of human individuals can present B29 to their T cells (and among RA patients possibly even more due to high presence of HLA-DRB1 *0401). In subsequent cultures they showed that 10 out of 14 healthy individuals could respond to the peptide. The B29 peptide was earlier tested in a mouse model of arthritis and it was found that prophylactic intranasal administration of B29 could suppress disease. Moreover, CD25+CD4+ T cells from B29 immunized mice could decrease disease severity in recipient arthritic mice, indicating that B29-specific Tregs are effective in diminishing autoimmune arthritis.

Next to the Treg inducing potential of B29, bone marrow derived dendritic cells pulsed with *Mycobacterium tuberculosis* (Mt) or mouse HSP70 induced IL-10
production in antigen-specific T cells and suppressed arthritis showing that HSP70 loading of DCs by itself is tolerising\textsuperscript{93}.

In order to make both tolDC therapy and HSP peptide treatment in autoimmune diseases (e.g. RA) as potent as possible, a combination therapy could be the solution. Pulsing tolDCs with HSP peptides could 1) solve the autoantigen problem and 2) the HSP peptides will be targeted to the HSP-specific T cells by DC with stable tolerogenic function, making sure a regulatory response towards the antigen is induced.

5. Conclusion

The fundamental problem in autoimmune diseases is the failure of the immune system to down-regulate its own potentially dangerous cells leading to destruction of tissue expressing the autoantigen. In the case of RA, currently available immunosuppressive therapies offer relief but fail to induce long-term physiological regulation resulting in medication-free remission.

As argued here, to restore immune tolerance, autologous tolDCs loaded with a HSP-derived peptide antigen could be used. Such a therapy could potentially both tolerise arthritogenic T cells and induce disease-suppressive regulatory T cells. Targeting the physiological mechanism of re-establishing tolerance for self-antigens offers the opportunity to inhibit joint-destroying immune responses long-term.

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\textbf{Conflict of interest}: 
WvE is shareholder of Trajectum Pharma, which develops immunotherapies on the basis of HSP70 peptide B29.

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References


93. Spiering R., van der Zee R., Wagenaar J., van Eden W., Broere F. Mycobacterial and mouse HSP70 have immuno-modulatory effects on dendritic cells.

Fig 1. HSP loaded tolerogenic dendritic cell (tolDC) vaccination in Rheumatoid arthritis (RA). This figure depicts the potential process that takes place in the patient’s joint after injection with HSP loaded tolDC. TolDC produce anti-inflammatory cytokines (e.g., IL-10) and present epitopes of HSP to naive CD4+ T cells. These CD4+ T cells differentiate into HSP specific Treg and suppress stressed (HSP expressing) cells via immunomodulatory cytokines like IL-10 and TGF-β. Furthermore, bystander suppression could lead to suppression of pathogenic Teff cells recognizing the unknown auto antigen, thereby inhibiting inflammatory symptoms. The presence of self hsp in the synovial fluid of RA patients might favor the selection of the generation of Treg and their function.

Table 1: HSPs and peptides associated with therapeutic interventions in chronic inflammatory diseases. dnaJP1 and DiaPep277 were tested in phase II clinical trials in juvenile RA and diabetes (refs. 85; 87). mB29a is now explored for the loading of tolDC in RA (refs. 83; 92). The peptides are based on human Hsp sequences.
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<thead>
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<th>HSP</th>
<th>Peptide</th>
<th>Sequence</th>
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<tr>
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<td>DiaPep277</td>
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