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Vaccines & Antibodies

DNA vaccines coding for heat-shock proteins (HSPs): tools for the activation of HSP-specific regulatory T cells

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Heat-shock proteins (HSPs) perform opposing functions in autoimmune arthritis. HSP-specific T cells drive the progression of adjuvant arthritis (AA), an experimental model of autoimmune arthritis. However, HSP-specific T cells can also have a regulatory phenotype, controlling arthritogenic T cells and inhibiting AA progression. This manuscript reviews the use of DNA vaccines coding for HSPs to analyse the role of these proteins in the regulation of arthritis. Recent studies suggest that HSPs participate in the control of pathological autoimmunity. Indeed, DNA vaccines coding for HSPs can be used to activate these HSP-specific built-in regulatory mechanisms. Thus, DNA vaccines coding for HSPs may serve not only as tools for the dissection of immunoregulatory mechanisms, but also as agents for the treatment of autoimmune disorders.

Keywords: arthritis, autoimmunity, DNA vaccination, heat-shock proteins

Expert Opin. Biol. Ther. (2005) 5(4):xxx-xxx

1. Introduction

Adjuvant arthritis (AA) models human rheumatoid arthritis (RA); the peripheral joint lesions in AA have most of the features that characterise RA, apart from their non-progressive nature [1]. AA is induced in Lewis rats by subcutaneous immunisation with heat-killed *Mycobacterium tuberculosis* (Mt) suspended in incomplete Freund's adjuvant [1]. Strikingly, humans exposed to high doses of mycobacteria, such as those in adjuvant immunotherapy for cancer, may also present a transient arthritis, in what could be considered as the human counterpart of AA [2].

AA is self-limited; the clinical manifestations of the disease become overt 12 days after immunisation with Mt and the severity of the disease increases over a period of 2 weeks, peaking around day 26. The active inflammatory process gradually subsides afterwards, but the swelling and anatomical deformities may last for a longer period [1]. Histological changes in the joints parallel the clinical manifestations of the disease. The peak of severe arthritis correlates with the detection of mononuclear cells and neutrophils in the articular cartilage. Late arthritis is characterised by fibrous ankylosis, caused by the proliferation of the pannus across the joint space [1].

AA induction with Mt triggers a strong T cell response to mycobacterial heat-shock proteins (HSPs) [3]; this T cell response is mainly mediated by Th1 cells that secrete IFN-γ and tumour necrosis factor (TNF)-α [4-8]. The mycobacterial 65-kDa HSP (HSP65) is a target of pathogenic T cells in AA; a T cell clone (A2b) specific for the epitope contained between amino acids 180 and 188 of HSP65 could adoptively transfer AA [9,10]. The A2b T cell clone can also react with cartilage proteoglycan, suggesting that targeting of inflammation to the joints might be due to crossreactivity between HSP65 and a self-component in cartilage [11].

HSP65 or some of its T cell epitopes can also induce resistance to AA when administered intraperitoneally, orally or via a recombinant vaccinia virus [3,12,13]. Inhibition of AA by treatment with HSP65 is thought to be mediated by regulatory T cells crossreactive with its mammalian counterpart, the 60-kDa HSP (HSP60) [14]. Regulatory T cells reactive with HSP60 have been isolated from both rats and humans [15-17]. Indeed, T cell reactivity to self-HSP60 is associated with a favourable prognosis in human RA [18] and juvenile chronic arthritis (JCA) [19,20].

Mycobacterial HSP65 therefore appears to provide epitopes with different immune functions in AA: the crossreaction of its 180-188 epitope with cartilage might drive the arthritogenic response, and the crossreactivity between HSP65 and self-HSP60 might regulate the disease. Other HSPs can also activate immunoregulation: the mycobacterial 10-kDa HSP (HSP10), the mycobacterial 71-kDa HSP (HSP71) or peptides derived from HSP70 can also protect against AA [8,21-24]. However, immunisation with other immunogenic bacterial proteins for which mammalian homologues are known does not influence the course of arthritis [25]. Consequently, different mycobacterial HSPs can control arthritic inflammation, but these anti-inflammatory properties of HSPs are not shared by other bacterial proteins highly conserved throughout evolution.

DNA vaccination is a method of immunisation that was initially used to induce protective immunity against infection and cancer; the mechanisms involved in the induction of a specific immune response by DNA vaccines have been reviewed elsewhere [26]. Briefly, following intramuscular injection, the DNA vaccine is taken up by local myocites [27] and dendritic cells [28]; the plasmid is maintained as an episome, which allows the expression of the encoded antigen [26]. The antigen is then secreted [29,30] and/or presented by myocites either at the site of injection [27] or by dendritic cells in the draining lymph nodes [28]. Thus, after a single or repeated injections of DNA, cellular and/or humoral immune responses to the encoded protein are mounted and long-lived memory lymphocytes are induced [26].

Several features render DNA vaccination an attractive tool to study the regulation of autoimmunity. Firstly, DNA immunisation is based on the synthesis of the antigen of interest within the host [26]. Therefore, DNA vaccines eliminate the need for tedious protein expression and purification procedures that can lead to the co-purification of undesired bacterial contaminants, such as lipopolysaccharide (LPS). LPS usually contaminates recombinant proteins expressed in bacteria; LPS has many effects on the immune system and has been shown to influence the course of experimental autoimmune disease [31-33]. Second, as DNA vaccines eliminate the need for antigen expression and purification, different constructs can be used to delete or add protein regions of interest and easily identify antigen domains associated with a particular immune effect. Third, the plasmids used in DNA vaccination contain immunostimulatory DNA sequences (CpG motifs) [34]. These CpG motifs trigger a series of responses in cells of both the innate and adaptive immune systems [35] via Toll-like receptor (TLR)9 [36], which boost the immunogenicity of DNA vaccines [37]. Thus, due to the ease of their production and re-engineering and the intrinsic adjuvant effects of CpG motifs, DNA vaccines might prove to be powerful tools to explore the complexity of the immune response. The safety of DNA vaccines in humans, however, has not yet been determined; until this is done, the clinical applications of DNA vaccination for autoimmune diseases and other conditions will have to wait. Indeed, it has been reported that vaccination with bacterial DNA might exacerbate, rather than control, some experimental models of autoimmune disorders [38]. However, the authors' experience vaccinating experimental animals with mammalian DNA encoding HSPs has, until now, been harmless if not beneficial.

The cross-talk between mycobacterial and self-HSPs in arthritis is not completely understood, neither are the anti-arthritogenic mechanisms triggered by HSPs understood. Can HSP-specific regulatory T cells be induced by vaccination? Do self-HSPs activate regulatory T cells? Which self-HSPs are immunoregulatory? And last but not least, what are the regulatory mechanisms involved? This paper will review how these, and other questions, were answered with the help of DNA vaccines coding for HSPs.

2. DNA vaccination with HSP65: DNA vaccines can activate HSP-specific regulation

Ragno and co-workers were the first to show that DNA vaccination with a construct coding for mycobacterial HSP65 (pHSP65) could arrest the progression of AA [39]. A detailed radiographic analysis revealed that pHSP65 DNA vaccination inhibited the joint narrowing and bone demineralisation that characterises AA. Histological examination revealed that pannus formation and fibrin deposition in the joint space were significantly diminished in pHSP65-vaccinated rats. These effects on the clinical signs of AA correlated with the induction of a strong humoral and cellular immune response to HSP65. Thus, DNA vaccination could trigger HSP65-specific T cells and ameliorate AA. Based on the high sequence homology existing between HSP65 and self-HSP60, pHSP65 vaccination might have arrested the arthritogenic process by inducing regulatory T cells reactive with self-HSP60. The HSP65-specific immune response, however, was not thoroughly analysed and, hence, crossreactivity with HSP60 and other potential regulatory mechanisms triggered by pHSP65-vaccination could not be evaluated in-depth.

3. DNA vaccines coding for HSP60: efficient inhibitors of adjuvant arthritis

Working with a recombinant vaccinia virus coding for human HSP60 (~ 95% homologous to rat HSP60), Lopez-Guerrero and co-workers had demonstrated that anti-HSP60 regulatory

T cells could be induced by vaccination and, therefore, either prevent [40] or treat [41] AA. Lopez-Guerrero's work was in agreement with previous studies that suggested that the protective effects of HSP65-specific T cell responses were associated with the induction of T cells crossreactive with self-HSP60 [3,42]. Indeed, a strong association has been found between the detection of T cells reactive with self-HSP60 and a better prognosis in JCA [19,20] and RA [18]. Could pHSP65 vaccination inhibit AA because of the induction of T cells crossreactive with self-HSP60? To answer this question the authors compared the effects on AA of DNA vaccination using constructs coding for HSP65 or HSP60 (pHSP60) [4]. It was found that both constructs could inhibit AA, but that pHSP60 was more effective than pHSP65. The immune effects associated with specific DNA-induced suppression of AA were complex and included enhanced T cell proliferation to a variety of disease-associated antigens. Effective vaccination with HSP60 or HSP65 DNA paradoxically led to upregulation of IFN-γ secretion to HSP60 and, concomitantly, to downregulation of IFN-γ secretion to the 180-188 epitope of HSP65. There were also variable changes in the profiles of IL-10 secretion to different antigens. However, vaccination with pHSP60 or pHSP65 enhanced the production of transforming growth factor (TGF)β1 to both HSP60 and HSP65 epitopes, suggesting that pHSP60 vaccination might have induced HSP60-specific regulatory T cells that shifted the Th1 arthritogenic response to a Th2/3 phenotype. The authors' results thus supported a regulatory role for HSP60 autoreactivity in AA. Furthermore, the data indicate that HSP60-specific regulation could be activated by DNA vaccination.

4. DNA vaccination with fragments of the HSP60 gene: identification of a regulatory epitope within HSP60

Several epitopes involved in the regulation of autoimmunity have been described within HSP60 [3,15,42-47]; many of these T cell epitopes are crossreactive with HSP65. To identify HSP60 regions involved in the regulation of AA by DNA vaccination, the authors divided the *hsp60* gene into five fragments and cloned each one of them into a separate vector suitable for DNA vaccination [5]. Groups of rats were vaccinated with each of the vectors, and the animals were challenged with Mt to induce AA. It was found that HSP60 DNA fragments could serve as effective vaccines against AA; vaccination with the N-terminal fragment of HSP60 coding for aa 1-140 (pI), or with the fragment coding for aa 130-260 (pII), led to a significant inhibition of AA. The 1-260 region was then examined using a library of overlapping HSP60 peptides to isolate regulatory epitopes. A regulatory peptide (Hu3) was identified that was specifically recognised by the T cells of pHSP60- or pI-vaccinated rats. Moreover, vaccination with Hu3, or transfer of splenocytes from Hu3-vaccinated rats, inhibited the development of AA. Vaccination with the mycobacterial homologue of Hu3 had no effect. Effective DNA or peptide vaccination was associated with enhanced T cell proliferation to a variety of disease-associated antigens, along with a Th2/3-like shift (downregulation of IFN-γ secretion and enhanced secretion of IL-10 and/or TGFβ1) in the response to the 180-188 epitope of HSP65. The regulatory response to HSP60 or to its Hu3 epitope included both Th1 (IFN-γ) and Th2/3 (IL-10/TGFβ1) secretors. These results therefore show that DNA vaccination with pHSP60 or pI activates HSP60-specific T cells responsive to regulatory self-HSP60 epitopes. Moreover, the authors' data show that DNA vaccines coding for HSP fragments can be used to identify new regulatory epitopes with potential therapeutic applications.

5. DNA vaccination with HSP70 and HSP90

Mycobacterial HSPs other than HSP65 can also control AA. Vaccination with the mycobacterial 10-kDa HSP or the 71-kDa HSP (HSP71) has been shown to inhibit the development of AA [8,21-24]; HSP71 has been shown to inhibit AA via the induction of T cells crossreactive with self-HSP70 [8,22]. Indeed, a mammalian member of the HSP70 family, BiP, is targeted by pathogenic T cells in AA and human RA, but can also be used to downregulate AA and collagen-induced arthritis [21,48]. Although immunoregulation of AA by mycobacterial HSPs different from HSP65 had been already described, little was known about the regulatory role of T cells reactive with self-HSP molecules other than HSP60. Hence, the authors studied the control of AA using DNA vaccines coding for the human 70-kDa (pHSP70) or 90-kDa (pHSP90) HSPs [4]. Both pHSP70 and pHSP90 downregulated the arthritogenic T cell response and inhibited AA. Vaccination with pHSP70 or pHSP90 led to a Th1 to Th2/3 shift in the response to Mt-derived antigens, including the 180-188 epitope in HSP65 that seems to drive the arthritogenic response. Thus, the pHSP70 and pHSP90 vaccines might inhibit AA by modulating the T cell response to Mt and the HSP65 180-88 epitope. BiP, a member of the HSP70 family of proteins, which has 64% homology with the HSP70 used in the authors' studies (accession number M11717), has also been reported to inhibit AA [21]. Remarkably, although they differ in their cellular localisation, both BiP and HSP70 are stress-inducible proteins. Therefore, it should be tested whether the immunomodulatory activity of HSP70 relies on its induction by stress. In conclusion, these studies demonstrated that DNA vaccines coding for self-HSPs other than HSP60 could also induce an antiarthritogenic immune response. However, the analysis of the HSP-specific T cells induced by pHSP70 or pHSP90 vaccination revealed unexpected findings about the regulatory responses triggered by DNA vaccination.

6. Connectivity of the immune response directed against different HSPs

DNA vaccination with pHSP70 or pHSP90 induces antigen-specific proliferative responses: pHSP70-vaccinated

rats manifest T cell responses to HSP70, and pHSP90-vaccinated rats manifest T cell responses to HSP90 [4]. However, DNA-vaccination with pHSP70 or pHSP90 could also induce T cells that proliferated and secreted IFN-γ, TGFβ1 and IL-10 following stimulation with HSP60 [4]. The cross-talk between the HSP60- and the HSP70-specific T cell responses is reciprocal: pHSP60-vaccinated rats showed significant T cell responses following stimulation with HSP70 [4], suggesting that different HSP molecules are immunologically connected.

To characterise this connection, the authors compared the epitope specificity of the HSP60-specific T cell response induced by pHSP60 with that induced by pHSP70 using a panel of overlapping peptides of human HSP60 [4]. The authors had previously found that pHSP60 DNA vaccination induced regulatory T cells reactive with a single HSP60 peptide epitope, Hu3 (aa 31-50) [5]. Although vaccination with pHSP70 induced strong reactivity to HSP60, the T cell epitopes targeted by the T cells of pHSP60- or pHSP70-vaccinated rats were different. pHSP60 vaccination induced a response to the Hu3 peptide alone, whereas pHSP70 vaccination induced responses to several HSP60 peptides: Hu19 (aa 271-290), Hu24 (aa 346-365), Hu25 (aa 361-380), Hu27 (aa 391-410), Hu28 (aa 406-425), Hu30 (aa 436-455), Hu32 (aa 466-485), Hu33 (aa 481-500) and Hu34 (aa 496-515) [4]. Thus, although both pHSP60 and pHSP70 can induce HSP60-specific T cells, the fine specificity of the T cell response induced by each DNA vaccine is different. One might wonder about the contribution of the HSP60-specific T cell responses triggered by pHSP70 or pHSP90 to the control of AA. In fact, several of the HSP60 peptides recognised by pHSP70-vaccinated rats contained AA regulatory T cell epitopes. Peptide Hu19 (271-290) partially overlaps with the peptide 283-297 described by Paul *et al.* (position relative to HSP60); T cells reactive with this region of HSP60 can inhibit AA [15]. In addition, three other HSP60 peptides targeted by the LNC of pHSP70-treated rats (Hu30: 436-455, Hu32: 466-485, Hu34: 496-515) overlap with a set of C-terminus HSP60 peptides described by Moudgil and co-workers (441-458, 469-483 and 491-507; position relative to HSP60) [46,47,49]. Vaccination with these HSP60 peptides, or transfer of peptide-specific T cells, also inhibited AA. Thus, the regulatory properties of HSP70 and HSP90 in AA might be reinforced by the induction of an immune response directed to regulatory HSP60 epitopes.

HSP60, HSP70 and HSP90 share no sequence homology and are not immunologically crossreactive (data not shown); it is therefore surprising that immunisation with pHSP70 or pHSP90 induces varying degrees of T cell response to HSP60. How could the immune responses to different self-HSPs be connected? One possible explanation for the induction of HSP60-specific T cell responses by pHSP70 or pHSP90 is self-vaccination with endogenous self-HSP60 induced and/or released as a result of the DNA vaccinations. Indeed, increased levels of HSP60 could be detected circulating in the serum of pHSP70-vaccinated rats [50]. The upregulation of HSP60 levels in the circulation was dependent on the presence of the *hsp70* gene on the pHSP70 DNA construct; a control empty plasmid had no effect on circulating HSP60 [50]. Reciprocally, vaccination with pHSP60 induced a T cell response to HSP70; however, the authors have not yet been able to measure the levels of HSP70 in the blood of pHSP60-vaccinated rats. As proteins encoded by DNA vaccines are detectable in the blood after vaccination [6,29,30] and HSPs are ligands for innate receptors [51], it is conceivable that HSP60, HSP70 and HSP90 mutually upregulate their expression via an innate receptor-mediated mechanism. This and other possible explanations for HSP immune cross-talk need further investigation; however, the finding mentioned above demonstrates that HSP-specific responses are inter-regulated and highlights the multiple immune signalling activities of HSP molecules. Finally, these results suggest that the immune responses to HSP60, HSP70 and HSP90 are connected by what might constitute a new type of crossreactivity.

The textbook definition of immunological crossreactivity cannot account for the HSP60/HSP70 crossreactivity seen here. For this reason, a second definition for crossreactivity is proposed. The authors define molecular crossreactivity as the classical crossreactivity that exists between antigens that share sequence or structural homology and are therefore recognised by the same T or B cell clones. This is the case, for example, for the crossreactivity existing between epitopes conserved in HSP65 and HSP60. Network crossreactivity, such as HSP60 and HSP70, is defined as the immune connection existing between molecules that bear no sequence or structural homology, but whose specific immune responses are interconnected (by self-vaccination or any other mechanism). Thus, network crossreactivity is a consequence of the organisation of the immune network; it does not rely on the existence of single T or B cell clones that recognise both antigens. The regulatory properties of HSPs may, therefore, result equally from the molecular crossreactivity existing between self and microbial HSPs, and from the network crossreactivity connecting different endogenous HSPs.

7. Immunoregulatory mechanisms triggered by HSPs

How do HSPs control inflammation? HSPs bear several features that might prove helpful for the control of the autoimmune response. HSPs are chaperones involved in the folding of newly synthesised proteins [52] and the processing and presentation of antigens by antigen-presenting cells (APCs) [53]. Circulating HSPs can also bind free peptides, facilitating their uptake and presentation by APCs through HSP-specific receptors [54-56]. In addition, circulating HSPs not loaded with any peptide can signal via innate receptors [51,57] and activate several cell types, such as dendritic cells [58-63], macrophages [64,65], T cells [66,67], B cells [68] and endothelial cells [69]. Finally, as described in this review, HSPs harbour regulatory T cell

epitopes. All of these observations suggest that the immunoregulatory effects of HSPs are based on complex interactions that involve several aspects of the immune response.

The picture gets more complicated when the expression of HSPs on different cell types and different conditions is analysed. The intracellular levels of HSP are increased under cellular stress [52]. Viral or bacterial infections upregulate HSP expression [70-72]. Bacterial DNA has recently been shown to trigger the secretion of HSPs [73], and necrotic cells, usually produced during the course of an infection, also release HSPs [63]. Inflammation is a source of cellular stress; HSPs are overexpressed at the sites of inflammation, such as in the synovium in arthritis [74]. HSPs are also upregulated in activated macrophages [75] and T cells [76], and, strikingly, HSP-specific T cells are activated in response to inflammation. Thus, during the course of an autoimmune response, HSP expression is upregulated at the target tissue and in the T cells carrying out the autoimmune attack.

Based on the known functions of HSPs and their intra- and extracellular localisation, one can foresee several mutually non-exclusive mechanisms involving adaptive and innate immunity that could contribute to the immunoregulatory properties of HSPs.

7.1 Innate immunity

7.1.1 Innate activation of regulatory T cells

HSPs are endogenous ligands for innate receptors [51,57]. HSP60 and HSP70 activate TLR4 and TLR2 [77,78], HSP70 and HSP90 have also been reported to signal via CD40 and CD91 [53,79,80]. Caramalho and colleagues have recently reported that regulatory $CD25^+$ T cells are activated via TLR4 [81]. Thus, it is possible that self-HSPs released to the circulation following DNA vaccination directly activate regulatory cells through innate receptors. This hypothesis is partially supported by the findings made in the authors' laboratory by Dr G Nussbaum, who generated non-obese diabetic (NOD) mice lacking a functional TLR4 ($TLR4^{-/-}$). $TLR4^{-/-}$ mice show an early onset and an increase in the incidence of spontaneous diabetes. Interestingly, the sensitivity of these NOD mice to cyclophosphamide-accelerated diabetes remains unchanged. As cyclophosphamide depletes regulatory cells [82], the accelerated diabetes seen in $TLR4^{-/-}$ mice suggests that TLR4-mediated signals triggered by self-ligands, such as HSPs, might activate regulatory cells involved in the control of autoimmune diabetes.

7.1.2 HSP60 triggers anti-inflammatory activities in T cells via TLR2

HSP60 and its p277 peptide (aa 437-460) can directly inhibit chemotaxis and activate anti-inflammatory activities in human T cells via TLR2 [67]. Human T cells activated in the presence of HSP60 also show a decreased secretion of IFN-γ and an increased secretion of IL-10. Thus, the HSP60 detected in the serum of pHSP60-vaccinated rats could control the activation of pathogenic T cells via TLR2.

7.2 Adaptive immunity

7.2.1 Environmental regulation of HSP-specific immunity

HSPs are immunodominant bacterial antigens [83]. As mucosal immunisation is known to induce antigen-specific regulatory responses [84], exposure to bacterial HSPs from the intestinal flora might be a source of HSP-specific regulatory T cells. Indeed, Moudgil and co-workers have demonstrated that environmental microbes can induce HSP65-specific T cells directed to regulatory epitopes crossreactive with self-HSP60 [46]. Inoculation with DNA vaccines coding for HSPs or their fragments might simply amplify this naturally acquired regulation.

7.2.2 Boost of regulatory T cell responses

HSPs can bind free peptides and induce peptide-specific immune responses, even in the presence of low amounts of the target peptide [54]. Thus, HSP molecules could be loaded *in vivo* with regulatory self-peptides and subsequently boost or amplify specific regulatory T cell responses. Indeed, Chandawarkar *et al.* reported that gp96 can both induce and downregulate tumour-specific immune responses [85]. This observation has been exploited as an immunotherapy: HSP70 purified from the inflamed CNS of experimental autoimmune encephalomyelitis (EAE) rats (and not from naive rats) can vaccinate naive rats against EAE [86]. Moreover, HSPs purified for the pancreas of diabetic mice or the CNS of mice with EAE could vaccinate against diabetes or EAE, respectively [87]. Thus, HSP–peptide complexes might be formed, including the circulating HSPs encoded by DNA vaccines, and trigger tissue-specific anti-inflammatory T cell responses [86].

7.2.3 Cytokine-mediated bystander inhibition

Inflammation leads to the upregulation of HSPs [74], and regulatory T cells reactive with self-HSPs have been purified from humans and rats [15-17]. Indeed, HSP-specific T cells that secrete regulatory cytokines (IL-10 and/or TGFβ1) are induced by vaccination with HSP-derived DNA vaccines or peptides [4-6,8,25]. HSP-specific regulatory T cells might be recruited to sites of inflammation where they could control pathogenic T cell clones by the secretion of regulatory cytokines.

7.2.4 Antiergotypic regulation

The authors have described a population of regulatory T cells that recognises antigens expressed by activated (but not resting) T cells, controlling their ability to induce experimental autoimmune disease [7,88,89]. The T cell receptor of these regulatory T cells recognises peptides derived from activation markers (ergotopes), such as the CD25 α-chain of the IL-2 receptor [7,88]. These cells are termed antiergotypic [89]. As HSPs are upregulated following T cell activation [76], they might serve as ergotopes targeted by regulatory antiergotypic T cells. The authors have studied whether vaccination with DNA vaccines encoding HSP60, or with the regulatory

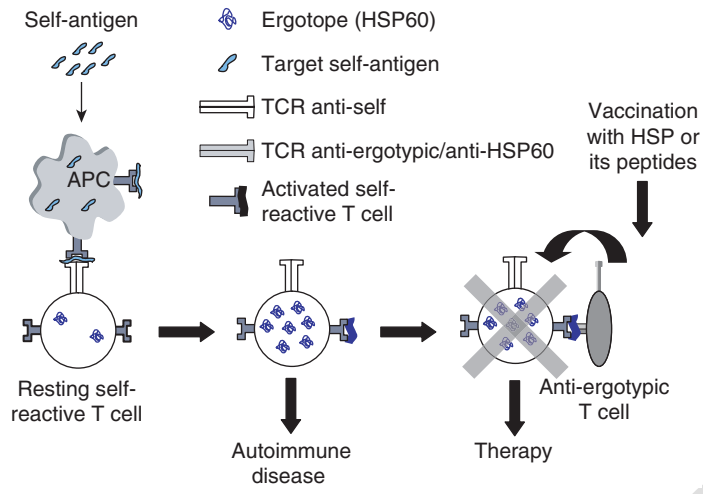

Figure 1. Antiergotypic response mediated by HSP60-specific T cells.
APC: Antigen-presenting cell; HSP: Heat-shock protein; TCR: T cell receptor.

peptide Hu3, might induce antiergotypic responses. To serve as an ergotope HSP60 must be upregulated in activated T cells, and activated T cells must present HSP60-derived peptides to HSP60-specific T cells.

T cell activation upregulates the mRNA coding for HSP60 as well as the HSP60 protein itself [76,90]. Rat and human T cells can function as APCs and, indeed, activated rat T cells present HSP60-derived T cell epitopes [90]. HSP60-specific T cells can be stimulated by activated T cells to proliferate and secrete IFN-γ and TGFβ1; this stimulation was MHC class II-restricted and required costimulatory signals mediated by CD80, CD86 and CD28 molecules (Quintana *et al.*, manuscript in preparation). Thus, HSP60 peptides are presented by activated T cells; however, are HSP60-specific antiergotypic T cells regulatory? To test this hypothesis the authors co-incubated antiergotypic HSP60-specific T cells with the lymph node cells of AA rats activated with the pathogenic 180-188 epitope from HSP65. Antiergotypic HSP60-specific T cells, but not control T cells, could inhibit the activation of arthritogenic T cells *in vitro* (Quintana *et al.*, manuscript in preparation). Moreover, when transferred into naive rats, antiergotypic HSP60-specific T cells could control the activation of arthritogenic T cells *in vivo*, preventing the induction of AA (Quintana *et al.*, manuscript in preparation).

Finally, are HSP60-specific antierogtypic responses induced *in vivo* by vaccination with DNA or peptides derived from HSPs? Vaccination with peptide Hu3 of HSP60 induced an MHC class II-restricted (RT1.B) antiergotypic T cell response (Quintana *et al.*, manuscript in preparation). However, DNA vaccination with pHSP60, or with vectors coding for its N-terminus fragments pI or pII, induced an MHC class II (RT1.B)- and MHC class I-restricted antiergotypic T cell response (Quintana *et al.*, manuscript in preparation). Moreover, DNA vaccines coding for HSP70 or HSP90 could also trigger HSP-specific antiergotypic responses. Thus, DNA and peptide vaccines derived from HSP60 can induce HSP60-specific $CD4^+$ and $CD8^+$ antiergotypic T cells *in vivo*. Antiergotypic regulation is therefore one of the mechanisms triggered by DNA vaccines coding for HSPs involved in the regulation of AA (**Figure 1**).

8. Expert opinion and conclusion

DNA vaccines coding for HSPs have taught us several lessons about the role of HSPs in the regulation of AA (**Figure 2**). First, DNA vaccines coding for HSP65 induce T cells reactive with self-HSP60. Second, HSP60-specific T cells have a regulatory phenotype in AA. Third, T cells reactive with HSPs other than HSP60, such as HSP70 and HSP90, can also control the arthritogenic reaction. Fourth, the T cell responses directed against different self-HSPs are connected via network crossreactivity; molecular crossreactivity exists between microbial and self-HSPs. Fifth, DNA vaccination with HSP60 or HSP70 induces T cell responses directed against different regulatory epitopes in HSP60. Sixth, HSP-derived DNA vaccines can regulate the progression of AA via complex mechanisms that involve both the innate and the adaptive immune responses.

The study of the anti-inflammatory mechanisms mediated by HSPs could lead to the design of novel therapies for autoimmunity – therapies to reinforce built-in HSP-based physiological mechanisms of control of immune function. Our results recommend the use of DNA vaccines coding for HSPs or their fragments for the activation of these regulatory mechanisms and the treatment of autoimmune arthritis, as well as for other autoimmune diseases. The arrest of the progression of EAE [4-6,39] and diabetes [82] with DNA vaccines coding for HSPs, together with the initial success of the HSP60 peptide p277 in treating human type 1 diabetes, show the feasibility of this approach [91].

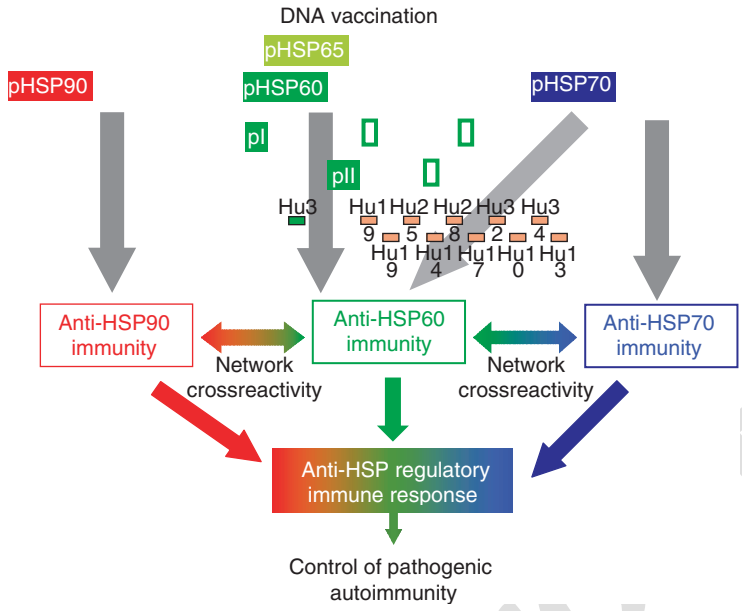

Figure 2. HSP-specific T cell responses induced by DNA vaccination.
APC: Antigen-presenting cell; HSP: Heat-shock protein.

Controlled autoreactivity is needed for the proper functioning of the immune system and body homeostasis [92], but might also be exploited for the design of new therapies for autoimmune disease. Thus, the combination of DNA vaccines coding for HSPs with methods aimed at the early detection of individuals at risk of developing autoimmune disease [93] might constitute new tools for the prevention and therapy of autoimmune disorders.

Acknowledgements

Professor Irun R Cohen is the incumbent of the Mauerberger Chair in Immunology, the Director of the Center for the Study of Emerging Diseases, Jerusalem, and the Director of the National Institute for Biotechnology in the Negev, at Ben-Gurion University of the Negev. The authors thank Ms Danielle Sabah-Israel for excellent secretarial assistance.

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