



HSP60: issues and insights on its therapeutic use as an immunoregulatory agent

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Heat shock proteins 60 (HSP60) is one of the most well studied member of the HSP family. Although found to be a target self antigen in pathological autoimmunity and HSP60-reactive T and B cells are part of immune responses in several infectious diseases, there is consistent experimental evidence that HSP60 displays dominant immunoregulatory properties. There are a series of reports on animal models showing that the administration of HSP60 can modulate inflammatory diseases. However, HSP60 has both immune-regulatory and inflammatory properties placing it as an essentially homeostatic antigen, but with potentially harmful effects as well. There have been a series of reports on the successful use of HSP60 and its peptides as immune-modulatory agent for several models of autoimmune diseases and in some clinical trials as well. We believe that the potential risks of HSP60 as a therapeutic agent can be controlled by addressing important factors determining its effects. These factors would be route of administration, appropriate peptides, time point of administration in the course of the disease, and possible association with other modulatory agents.

Keywords: heat shock proteins, HSP60, homeostasis, inflammation, immunoregulation

HSP60 AS AN IMMUNODOMINANT HOMEOSTATIC ANTIGEN

Heat shock proteins (HSP) are highly conserved proteins during evolution. Besides being upregulated in stress conditions, they are constitutively and abundantly present in all living beings and are profoundly involved in various intracellular and systemic homeostatic functions in different species (Hemmingsen et al., 1988; Karlin and Brocchieri, 2000). This feature can be illustrated by their capacity to prevent protein degradation in salamanders or in tropical trees that live exposed to very high temperatures (Kusukawa and Yura, 1988), or by their capacity to interact with the immune system in mammals, contributing to the fine tuning of inflammation (Pockley et al., 2009), and participating in cell survival signaling pathways (Chun et al., 2010). These robust homeostatic properties and their marked evolutionary conservation lead us to think that HSP can be considered as dominant homeostatic molecules in evolution. Taken the diversity of HSP families (classified according to their molecular weight), it is possible that different HSP families play differential roles in the complex integrated homeostatic network.

Heat shock proteins 60 is one of the most well studied HSP, especially in the interaction with the immune system. It was first described as intracellular chaperone, assisting the folding of polypeptides into proteins and the transporting of proteins (Bukau et al., 2006), but later it was found to be a dominant antigen recognized during infections, together with HSP70 (Kaufmann, 1990; Zügel and Kaufmann, 1999). These properties place these two proteins in a special category as antigens. Indeed, HSP60 has been intensely studied in various immunologic contexts such as in autoimmune diseases (Danielle et al., 1992; Tanaka et al., 1999;

Dieude et al., 2004; Quintana et al., 2004, 2008; Van Eden et al., 2005) – often considered as an important target molecule in the pathogenesis – in tumors (Michaluart et al., 2008; Vitorica et al., 2009) and in transplantation (Qian et al., 1995; Birk et al., 1999; Granja et al., 2004). But, HSP60 does not seem to be a relevant antigen only in pathological conditions. Autoreactivity to HSP60 is also found in health and a high frequency of B and T cell clones reactive to self HSP60 is found in physiological conditions in mammals (Munk et al., 1989; Fischer et al., 1992; Abulafia-Lapid et al., 1999; Pockley et al., 1999).

Heat shock proteins have been considered by Irun Cohen (Cohen and Young, 1991) as immunodominant antigens for self immune responses and as key players in physiological autoimmunity, likely to be involved in homeostasis (Cohen and Lohse, 1991; Cohen, 1992). In line with this idea, self-HSP-reactive T and B cell clones can be seen as an important part of a network of regulatory cells and molecules in the immune system engaged in homeostatic activities (Cohen, 2007). These activities would include tissue maintenance and repair, but also limiting clonal expansion and controlling inflammation.

Since the discovery of AIRE and its role in the expression of various tissue specific antigens by thymic medullary epithelial cells (MEC; Anderson et al., 2002), it became clear that the different levels of expression of these antigens placed them at a hierarchical order of relevance for central tolerance (Kyewski and Klein, 2006). All highly expressed antigens would compose a particular set of “dominant” antigens relevant for the immune homeostasis (Coutinho and Haas, 2001). In this sense, HSPs can be considered as strong candidates to be part of this immunodominant

homeostatic self. They are ubiquitous antigens expressed both in housekeeping as well as in stressed circumstances, and they are highly expressed in the thymus (Mamalaki et al., 1996). Taken that transgenic expression of HSP60 in the thymus does not lead to the deletion of HSP60 specific T cell clones (Minter and Osborne, 2003), it is possible that central tolerance to HSPs relies on the generation of natural regulatory T cells (nTregs) akin to the way tolerance is imposed to many other self antigens (Coutinho et al., 2005; Aschenbrenner et al., 2007). Indeed, nTregs do recognize self antigens and HSPs, including HSP60, are likely to be among these self antigens. In addition, HSP60 has a strong effect in the survival and function of CD4+CD25+Foxp3+ regulatory T cells (Zanin-Zhorov et al., 2006) and they were shown to efficiently drive the differentiation of CD4+CD25- T cell clones derived from Juvenile idiopathic arthritis (JIA) patients into CD4+CD25^{high} regulatory T cells expressing GITR, CTLA-4, and Foxp3 (de Kleer et al., 2004).

In addition to the reported immunomodulatory effect of HSP60, it is clear that this molecule also displays inflammatory actions. HSP60 activates both innate and adaptive arms of the immune response being considered as a danger signal that amplifies inflammation and influence a wide range of immune reactions (Chen et al., 1999; Wallin et al., 2002; Habich and Burkat, 2007). Anti-HSP antibodies and HSP60-reactive T cells are part of immune responses in several infectious diseases (Kaufmann, 1990). Approximately 10–20% of the specific T cells in mice immunized with *Mycobacterium tuberculosis* are against the bacterial HSP65 (Kaufmann et al., 1987). Antibodies to HSP60 of *Chlamydia trachomatis* have been detected at high levels in the sera of infected patients (Sanchez-Campillo et al., 1999), and immunodominant responses to HSP60 are present in other fungal infections (Matthews et al., 1998). This strong immune response directed to HSP60 during infection can be explained by its critical role in cellular homeostasis and by its upregulation in host tissues as a result of stress during infection. In addition, antibodies as well as effector pathological T cells reactive to self HSP60 were found in different autoimmune and inflammatory diseases including type 1 diabetes (Elias et al., 1991; Birk et al., 1996; Abulafia-Lapid et al., 1999) rheumatoid arthritis (RA), multiple sclerosis (Quintana et al., 2008), lupus erythematosus (Dieude et al., 2004), atherosclerosis (Xu et al., 1993), Behcet's disease (Tanaka et al., 1999), inflammatory bowel disease (Stevens et al., 1992), and inflammatory skin disorders (Bayramgurler et al., 2004).

Although HSP60 was first found to be a mitochondrial protein (McMullin and Hallberg, 1988), it is now known that, in eukaryotes, it can also locate in the cytosol (Chandra et al., 2007), at the cell surface (Wand-Wurttenberger et al., 1991; Soltys and Gupta, 1997), in the extracellular space (Davies et al., 2006), and soluble in the peripheral blood (Cappello et al., 2008). In addition, HSP60 peptides can be also be presented in MHC class I and class II molecules (Koga et al., 1989; Anderton et al., 1995), activating specific CD8+ and CD4+ T cells (Van Eden et al., 2005). Thus, we believe that many of its immunologic functions may be triggered/modulated by soluble HSP60 and its peptides present in the microenvironment. In this respect, several synthetic HSP60 peptides have been shown to be immunologically active *in vitro* (Paul et al., 2000; Caldas et al., 2004; Van Eden et al., 2005) and *in vivo*

(Elias and Cohen, 1995; Huurman et al., 2007), but the repertoire of HSP60 peptides actually generated *in vivo* in different physiological and pathological contexts is still unknown.

Immunologic activation induced by any type of stimulus triggers a variety of both inflammatory and anti-inflammatory mechanisms, and the capacity to both induce and control inflammation is critical to homeostasis. HSP60 has both properties placing it as an essentially homeostatic antigen, but with potentially harmful effects as well. Thus, we think it is crucial to always look at both functional sides of the immune response in any given immunologic context. We have proposed the term REG/INFLAMMA to denote functional activities predominantly immunoregulatory or proinflammatory of any given immune molecule. In our descriptive model, a REG/INFLAMMA molecular panel of immunologically relevant transcription factors (such as GATA-3, ROR γ t, T-bet, Foxp3) and cytokines (such as IL-6, TGF- β , IL-10, IFN- γ , TNF- α) is used as a way to evaluate the overall immunologic activity of a given molecule or of a particular pathological context (Moraes-Vieira et al., 2012). We believe this may be a useful strategy to evaluate the functional activities of HSP60 peptides, for selecting adequate candidates for clinical application. As a matter of fact, for the specific selection of HSP60 peptides, the REG/INFLAMMA panel could be expanded to also include relevant innate molecules, since HSP60 and its derived peptides also interact with a variety of these molecules.

Some of the HSP60-autoreactive T cell clones have been already isolated from mice (Birk et al., 1996), rats (Feige and Cohen, 1991), and humans (Caldas et al., 2006), under a variety of inflammatory conditions, showed REG and INFLAMMA properties *in vitro* and *in vivo*. This dual immunologic activity places Hsp60 and its derived peptides in a privileged position as potential therapeutic immunomodulators, to either amplify or control inflammation. On the other hand, the same duality also poses a concern for clinical application because it is still unclear what determines a predominant REG or INFLAMMA outcome induced by HSP60 and derived peptides, as previously discussed (Coelho et al., 2008).

HSP60 REGULATORY ACTIVITY IN INFLAMMATORY DISEASES

Although found to be a target self antigen in pathological autoimmunity, there is consistent experimental evidence that HSP60 displays dominant immunoregulatory properties. There are a series of reports on animal models showing that the administration of HSP60 can generate T cell responses that modulate inflammatory diseases (arthritis, type 1 diabetes, atherosclerosis, EAE, asthma, lupus, dermatomyositis; Quintana and Cohen, 2011).

Administration of HSP60 and its peptides by oral, nasal, intraperitoneal, and subcutaneous routes has been extensively studied in experimental models of arthritis in rats (Van Eden et al., 2005). The protective effects of these protocols lead some groups to explore the reactivity of T cells of patients with JIA and RA to HSP60. Interestingly, HSP60-reactive T cells isolated from JIA, but not from AR, patients display a regulatory phenotype and secrete predominantly cytokines such as IL-10 e TGF-beta (de Kleer et al., 2004).

Intranasal and oral administration of HSP65 from *Mycobacterium tuberculosis* has been also tested with success in the

Ldlr^{-/-} mice which develop atherosclerosis upon feeding a hypercholesterolemic diet (Harats et al., 2002; Maron et al., 2002). HSP65-treated mice showed a co-relation between plaque reduction and IL-10 expression at the aortic arch (Maron et al., 2002).

In the NOD mice, a type 1 diabetes model, systemic treatment with HSP65 from *Mycobacteria* as well as with HSP60 p277 peptide trigger immunoregulatory pathways that result in suppression of disease (Elias et al., 1991; Elias and Cohen, 1995; Birk et al., 1996). Phases II and III clinical trials using HSP60 p277 in patients with type 1 diabetes have been successful in the preservation of islet beta-cell function (Raz et al., 2001, 2007) and, more recently, also in a randomized phase III trial with over 400 newly diagnosed type 1 diabetes patients. Other clinical trials employing HSP60 peptides arrested the autoimmune destruction in patients with RA (van Roon et al., 1997; de Kleer et al., 2004), and autoimmune uveitis (Stanford et al., 2004).

The capacity of HSP60 to control inflammation in the context of transplantation seems to be a harder task. May be this is related to the more robust inflammatory response, simultaneously triggered by multiple alloantigens, in an already highly inflammatory milieu induced by surgery, in contrast to the more insidious development of inflammation in autoimmune diseases, together with the insidious occurrence of epitope spreading to newly exposed self antigens. Nonetheless, our group has been investing on the identification of potent HSP60 immunoregulatory peptides for use in allotransplantation. We have been able to prolong allograft survival across both minor (Luna et al., 2007) and major (unpublished data from V. Coelho laboratory) alloantigen disparities but so far, we have been unsuccessful in inducing transplantation tolerance. Nevertheless, other investigators have been able to induce transplantation tolerance in a mouse model of skin allograft (Birk et al., 1999), but publications in this field are scarcer. Despite the difficulties, we believe it is worth investing on the use of HSP60 peptides as immunomodulators in allotransplantation; may be not alone, but in combination with other drugs and immunoregulatory molecules. If HSP60 and its peptides are, indeed, endogenous immunoregulators, we should find ways to enhance/activate endogenous immunoregulatory networks, in a synergic manner with other therapies. This strategy seems particularly relevant to transplantation, due to the multicity of antigens involved in triggering inflammation; each pair of donor/recipient comprise a different set of alloantigens inducing aggression against the graft. If we are able to identify combinations of conserved dominantly immunoregulator HSP60 peptides, they could be used in allotransplantation, alone or in combination with other immunoregulatory therapies, irrespective of specific alloantigen disparities. A broad immunoregulatory effect is likely to occur once human HSP60 DNA vaccination has been able to control disease in NOD diabetes, inducing a Th2-like immune response not only to HSP60 but also to other relevant target self antigens, such as GAD (glutamic acid decarboxylase) and insulin (Quintana et al., 2002).

IMMUNOREGULATORY MECHANISMS INDUCED BY HSP60

Heat shock proteins 60 and derived peptides are able to exert an important role in the fine balance between promoting and controlling inflammation through a variety of mechanisms. It is very likely

even that HSP60's REG and INFLAMMA functional activities occur simultaneously within an inflammatory microenvironment, probably also mediated by its different peptides, affecting different cell types, and mobilizing different molecular pathways. However, it is not known whether specific molecular signals, either immune cell or tissue-derived, influence HSP60's functional activity in the course of inflammation. Maybe there is also a time course difference – a proinflammatory predominance at the initial phase of inflammation and a regulatory one at a later time point – as we previously suggested in the context of human renal transplantation (Granja et al., 2004).

Heat shock proteins 60 can be viewed as a protein antigen that will be processed and presented to T cells inducing a typical lymphocyte reaction. However, HSP60 and its various peptides have the capacity to interact with a variety of immune molecules, intracellularly (Cappello et al., 2008; Chun et al., 2010) and at the cell surface such as with TLR2, TLR4 (Vabulas et al., 2001), and MHC molecules (Newcomb and Cresswell, 1993), bridging innate and adaptive immune responses. Therefore, plasticity and multiple connectivity are also striking immunologic properties of HSP60.

An important issue that may be raised regarding this plasticity is the relationship between the dual role (regulatory and inflammatory) of HSP60 with its binding molecules. The signaling of an endogenous protein expressed in low amounts chronically may lead to distinct patterns of activation of TRL-expressing cells such as dendritic cells, macrophages, and T cells. In this respect, we would like to highlight the complex diversity of effects triggered by different molecules through the same receptor on different cell types. For instance, it has been shown that both HSP60 and HSP70 signaling, through TLR2 and NF- κ B activation, on murine cardiomyocytes induce, *in vitro*, contractile dysfunction and cell death (Mathur et al., 2011). The treatment of human CD4⁺CD25⁺ Tregs with human HSP60 or its p277 peptide before anti-CD3 activation also enhanced their ability to down-regulate proliferation and production of IFN- γ and TNF- α by CD4⁺CD25⁻ or CD8⁺ target T cells. In addition, the enhancing effects of HSP60 costimulation on Tregs involved innate signaling via TLR2, led to activation of PKC, PI3K, and p38, and were further enhanced by the inhibition of ERK. HSP60-treated Tregs suppressed target T cells both by cell-to-cell contact and by secretion of TGF- β and IL-10 (Zanin-Zhorov et al., 2006). HSP60 is also a ligand that activates B cells via TLR4. Simultaneous ligation of TLR4 and BCR in HSP60 specific B cells induces antibody secretion (Cohen-Sfady et al., 2005; Herlands et al., 2008) and this may be an important way to induce the anti-HSP60 IgM autoantibodies that are prevalent in physiologic conditions (Merbl et al., 2007). TLR4 ligation in epithelial cells are also known to play a protective role in the intestinal mucosa. On the other hand, high concentrations of HSP60 usually trigger TLR4 signaling and inflammatory activation of monocytes (Pockley et al., 2009).

Adding more complexity to the plethoric immunobiology of HSP60, it was recently reported that HSP60 interacts with beta-catenin, increasing its transcriptional activity and protein expression, and favoring metastasis (Tsai et al., 2009). Taken that beta-catenin also enhances cell survival of several cell types including Tregs (Ding et al., 2008), it is plausible that this may represent an

additional pathway by which HSP60 and derived peptides promote immunoregulatory activity.

THERAPEUTIC USE OF HSP60 FOR INFLAMMATORY DISEASES: ADVANTAGES AND RISKS

Since administration of Hsp60 has a well documented regulatory effect in inflammatory disease models, it has been proposed by several groups that this antigen may be used as a therapeutic tool in diseases where inflammation is undesirable such as autoimmune and allergic diseases and transplantation.

Despite the concerns about the potential risks of inducing inflammatory undesirable effects, different formulations of HSP60 and derived peptides have been used for immunoregulation in a variety of animal models, and no evidence of pathological autoimmunity was detected. Accordingly, we have not found histopathological signs of inflammation in over 10 tissues following the use of the HSP65-DNA vaccine, in mice (Lima et al., 2009). Nevertheless, we believe that the use of HSP60 peptides displaying a predominant immunoregulatory function, instead of the whole molecule, may reduce potentially harmful effects. In addition, this strategy may provide an opportunity to combine different peptides which trigger distinct immunoregulatory mechanisms. Nonetheless, depending on the route of administration, the quantity of peptides required may be a limiting issue for clinical application.

Another risk that can be foreseen for the use of HSP60 is a possibility of immunosuppression to protective responses against infection. Experiments carried out by our group have shown that mice treated with HSP65 by the oral route for 4 days have decrease immune responses to HSP65 but show a normal response to *Salmonella* infection (Rezende et al., unpublished results). The course of infection was not altered by the treatment suggesting that protective responses to infectious agents may rely on other potent pathogen-associated antigens.

On the contrary, in infectious contexts, immune response to bacterial antigens might benefit from the fact that there is a high frequency of self-HSP-reactive T and B cell clones in a normal repertoire. These HSP-reactive lymphocytes would be recruited and take part in the ongoing immune response, exerting mainly a regulatory role and contribute to reestablishing homeostasis (Cohen and Young, 1991). Indeed, immunomodulation of inflammatory responses during infection is critical for the outcome of the infectious disease. There are several examples of infectious diseases in which severity of disease is mostly related to a high prevalence of proinflammatory cytokines (especially TNF- α) and poor immune-regulatory components (such as IL-10 or Tregs). This is true for Chagas disease (Vitelli-Avelar et al., 2008), Schistosomiasis (Wamachi et al., 2004), and Leishmaniasis (Oliveira et al., 2011).

It has been shown that effector CD4⁺ T cells that secrete large amounts of IL-2 are able to efficiently recruit CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Curotto de Lafaille et al., 2004; Almeida et al., 2006). IL-2 is a critical cytokine for Treg function and this is one of the mechanisms triggered during inflammatory events capable of modulating the degree of tissue damage without compromising needed inflammatory responses. Therefore, the REG/INFLAMMA balance is an essential part of all inflammatory responses. HSPs fit very well to this picture since

they are self proteins ubiquitously expressed and also they are upregulated in stress conditions.

The fact that HSP60 is an ubiquitous molecule, on the other hand, may represent another benefit. Its use would circumvent the need for the identification of the target antigens involved in the induction of each inflammatory disease. In this regard, it has been demonstrated that tolerance induced to an antigen can recruit regulatory T cells and molecules that would spread their effect in a bystander fashion (Miller et al., 1991). This bystander effect depends on the antigen presenting cells that induce the regulatory T cells and it recruits for the modulatory immune network most the antigens presented in the same context.

In view of these potential risks and benefits, we believe that some issues have to be addressed on the therapeutic use of HSP60 as an immunoregulatory agent: route of administration, form of the protein (whole molecule, peptides), time point in the course of the disease, possible association with other modulatory agents.

ROUTE OF ADMINISTRATION

The majority of studies on the use of HSPs and HSP60 as modulatory agents in several models of inflammatory diseases have used the parenteral (subcutaneous, intraperitoneal, intravenous, and intramuscular) route of administration (Van Eden et al., 2005), probably because of its easy translation into a later clinical setting. However, we believe it is time to reexamine the pros and cons of the routes in the light of new knowledge on HSP60 immunobiology.

It has been extensively described that the oral route is a very efficient way to induce peripheral tolerance in animal models (Faria and Weiner, 2006) and also in humans (Mestecky et al., 1996). Oral tolerance is a well known phenomenon that probably accounts for the robust balance that keeps the homeostasis of the gut mucosa to the daily challenge of microbiota and dietary antigens (Faria and Weiner, 2005). Indeed, we are all tolerant to the food proteins we ingest and also to our microbiota and this has been documented in mice and humans (Andrade et al., 2006; Round et al., 2010). This is especially interesting regarding HSP because bacterial components of our microbiota do express HSPs and they are likely to be involved in immunoregulatory networks in the gut. Although many of these proteins are intracellular, we now know that HSP60 can also be expressed at the cell surface and in the extra cellular space, therefore providing a variety of molecular forms and pathways by which HSP60 could affect the immunologic milieu in the gut. In addition, it is plausible that some of the luminal contents (including dead bacteria) in contact with the abundant lymphoid tissue of the gut mucosa would induce regulatory T cells and oral tolerance. Therefore, oral tolerance to our microbiota can be envisaged as a peripheral form of homeostasis reinforcement of tolerance involving HSPs.

Of all the feeding regimens already tested for oral tolerance, continuous feeding has been shown to be the most efficient way to induce tolerance. Continuous feeding of antigen, but not gavage, can render otherwise refractory animals, such as aged mice, tolerant. Moreover, oral tolerance induced by continuous feeding of antigen lasts longer and, in mice, do not require any type of reinforcement during their lifetime (Faria et al., 1998, 2003).

Since continuous feeding protocols are very cumbersome for human studies, the use of a probiotic vehicle has been designed to continuously deliver *Mycobacterium leprae* HSP65 in the gut mucosa upon a single administration daily. This strategy associates the immunomodulatory and tolerogenic potential of HSP65 and of the gut mucosa. We observed that oral administration of Hsp65-producing *Lactococcus lactis* prevented EAE in mice (Rezende et al., unpublished results). Histological analysis showed no inflammatory cell infiltrate and no tissue destruction in the spinal cord of HSP65-treated mice. Moreover, increased frequency of regulatory T cells correlated with the treatment. Thus, this strategy may constitute a promising alternative therapy for the treatment of autoimmune and inflammatory diseases.

Nasal administration of antigens is also an effective form of inducing long living tolerance (Mestecky et al., 1996; Faria and Weiner, 2006). Regulatory elements triggered by nasal versus oral administration of antigen may be distinct but both routes have been described as efficient ways to modulate inflammatory diseases by HSP60, including reducing atherosclerotic plaque formation in the *Ldlr*^{-/-} mice (Maron et al., 2002) and prolonging allograft survival in mice (Luna et al., 2007). We would like to point that other forms of continuous delivery of HSP60 have been explored with success. Accordingly, intranasal administration of HSP60 peptide p277 encapsulated into polylactide-co-glycolide acid microspheres resulted in increased skin graft survival in two combinations of murine strains with minor H-2 disparities (Luna et al., 2007).

We believe that the use of strategies such as continuous delivery of antigen by mucosal routes is an appropriate way to favor anti-inflammatory activities of HSPs by mimicking natural routes of tolerance induction and should be further explored.

WHOLE PROTEIN OR PEPTIDES?

An important caveat regarding the *in vitro* functional studies using recombinant HSP60 is endotoxin contamination which, even at very low concentrations, may still influence cytokine production and immune cell functional activity. This seems particularly important because HSP60 and its derived peptides bind to TLRs, including TLR4 which is also a ligand to endotoxin, raising controversies regarding HSP60 specific immunologic effects. This issue has been extensively discussed in the literature and several investigators claim that there is sufficient evidence to support HSP60 specific signaling actions (Henderson et al., 2010). Indeed, working with low-endotoxin HSP60 recombinant preparations has minimized the effect of bacterial contaminants. However, we believe this issue will only be clearly solved when HSP60 produced in endotoxin-free systems is used for functional assays. Taking this into consideration, as well as HSP60 REG/INFLAMMA properties, several groups and ours have been exploring the immunologic functions of HSP60 synthetic peptides in a variety of pathological and physiological (Prakken et al., 1997; Luna et al., 2007) contexts, and have identified immunologically active peptides, effective in, at least partially, downregulating inflammation in animal models (Elias et al., 1991; Thompson et al., 1998; Luna et al., 2007) and in humans in the context of diabetes (Raz et al., 2001, 2007).

Using a panel of HSP60 synthetic peptides and a gene panel of predominantly REG/INFLAMMA molecules, we have found several peptides displaying the capacity to simultaneously modify the gene expression of a variety of immune molecules, *in vitro*, and have identified some predominantly REG and others INFLAMMA, using both mouse splenocytes and human peripheral blood mononuclear cells (PBMC; unpublished data from V. Coelho's laboratory). Interestingly, some predominantly regulatory HSP60 peptides were shared by the two species, suggesting evolutionary functional conservation.

TIME POINT OF THE DISEASE

For oral administration protocols, it is well established that the regulatory mechanisms of oral tolerance is very efficiently triggered before inflammation onset and their efficiency declines progressively after sensitization. Therefore, for oral administration of hsp65, early diagnosis and treatment would be the ideal protocol. This will be critical for HSP60 administration considering the dual functional properties of the antigen (REG-INFLAMMA). Another way to circumvent this caveat of oral treatment with HSP60 might be the association with a delivery system that could act as a modulatory adjuvant boosting the tolerogenic effect of HSP60.

As mentioned before, it is not known whether specific molecular signals from the tissues influence HSP60's functional activity in the course of inflammation. It is possible that a time course difference exists as we have observed in patients who had renal transplants (Granja et al., 2004). Autoreactivity to HSP60 displayed a proinflammatory effect at the initial phase of transplantation and a regulatory one at a later time point.

ASSOCIATION WITH OTHER MODULATORY AGENTS

Since immune tolerance mediated by HSP60 could be more difficult to induce in sensitized diseased individuals, the use of modulatory adjuvants might be helpful. If the oral route is chosen, probiotic bacteria would be excellent candidates since they are well known as agents with immunomodulatory properties. Several probiotic bacteria are already in use for this purpose (*Lactobacillus*, *Bifidobacteria*, *Lactococcus*) and the ones that were genetically engineered to express immune-relevant proteins are specially interesting (Pontes et al., 2011). A trial on IL-10-producing *L. lactis* is currently being carried out after promising results in animals models of inflammatory bowel disease (Braat et al., 2006). Hsp65-producing *L. lactis* would also fulfill these requirements since the bacteria *L. lactis* has modulatory properties on its own (unpublished data) besides their effect as delivery agent.

Another interesting strategy recently reported is the use of a pathologically relevant antigen along with HSP60 to induce immunomodulation. Nemirovsky and coworkers used amyloid beta peptide A β 1-15 conjugated with HSP60 peptide p458 in a subcutaneous vaccination protocol for a murine experimental model of Alzheimer disease. The combined peptide vaccine resulted in a significant decrease in cerebral amyloid burden and inflammation in the brain (Nemirovsky et al., 2011). A similar approach could be designed for autoimmune diseases such as type 1 diabetes, arthritis, myasthenia gravis, and others for which a target self molecule has already been identified.

CONCLUDING REMARKS

The dual immunologic role of HSP60 and derived peptides – inflammatory and regulatory – can be viewed as an advantage for multiple clinical applications, either boosting effector responses or controlling inflammation. On the other hand, it also raises concerns, since it is still unclear what determines HSP60's

immune-regulatory or inflammatory activities. We believe that critical factors such as route of administration, time course of the disease in which it will be used, dosage, HSP60 peptides chosen, and the combination with other modulatory molecules may circumvent the putative risks posed by its inflammatory potential.

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