HSP70 Is a Major Contributor to the MHCII Ligandome and Inducer of Regulatory T Cells

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Abstract Experimental models of autoimmunity have revealed anti-inflammatory effects of immunization with HSP70 or its derivative peptides. In depth cellular analysis of the effects of HSP70 immunization has shown the capacity of HSP70 to induce and expand self-tolerance promoting regulatory T cells (Tregs). In other words, in the models tolerance was re-established by the action of HSP70 specific Tregs. For the inflammation suppressive activity of antigen specific Tregs it is essential that the targeted antigen is ubiquitously expressed in the tissues. HSP70 family members, especially those that are stress-inducible, are widely expressed by stressed cells in the inflamed tissue due to the local presence of inflammatory mediators. In addition, cell stress is known to lead to autophagy, which in the case of chaperone mediated autophagy does lead to the preferential loading of HSP70 in MHC class II molecules. MHCII peptide elution profiles obtained from cells in a steady state have also revealed the dominating presence of HSP70 derived peptides in MHC class II molecules. For these reasons HSP70 is one of the most frequent cytosolic/nuclear MHCII natural ligand sources. HSP70, when presented by tolerizing antigen presenting cells in tissues, does induce Tregs, which seem to contribute to the tolerance promoting default setting of the healthy immune system.

Keywords Autoimmunity · Hsp70 · MHC · Peptide · Tolerance · Treg

Abbreviations

BMDC	bone marrow derived dendritic cells
CMA	chaperone mediated autophagy
DC	dendritic cells
ER	endoplasmic reticulum
ERK	extracellular signal regulated kinase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase

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IL-10	interleukin 10
JNK	c-jun N-terminal kinase
LAG-3	lymphocyte activating gene-3
MAPK	mitogen activated protein kinase
MHC	major histocompatibility complex
MS	multiple sclerosis
NFkB	nuclear factor kappa beta
OVA	ovalbumin
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PGIA	proteoglycan induced arthritis
RA	rheumatoid arthritis
TcR	T cell receptor
TNF	tumor necrosis factor
tolDC	tolerized dendritic cells
Treg	regulatory T cells

Introduction

The first observations showing the disease suppressive effects of HSP in autoimmune disease models were made in the model of adjuvant arthritis in rats (van Eden et al. 1988). Since then the effects of HSP immunizations were analyzed in many different disease models, amongst which were arthritis, diabetes, EAE and allergies. Almost without exception HSP was found to prevent or diminish the expression disease (reviewed by (van Eden et al. 2005). In a study by Anderton et al. (1995) it was shown that mycobacterial HSP60 was protective in adjuvant arthritis due to the induction of a regulatory response of T cells that cross-recognized the mammalian HSP60, as expressed in the tissues. By this and subsequent studies it was shown that due to their evolutionary conservation, microbial HSP were triggering self-HSP recognizing T cells with IL-10 mediated anti-inflammatory activities. In addition to this, when it was shown that other conserved and immunogenic microbial proteins had no disease inhibitory effect in the models, it became apparent that HSP were exceptional in this quality of modulating inflammatory diseases (Prakken et al. 2001). It is possible that besides conservation, also the stress inducibility in tissue adds to the unique features of HSP as disease modulators.

HSP70 and Its Anti-inflammatory Effects in Experimental Models

For HSP70, disease suppressive effects in adjuvant arthritis were seen with *M. tuberculosis* derived recombinant HSP70. Again, a conserved mycobacterial HSP70 sequence was found to be immunogenic and to induce T cells that cross-reacted

with the rat homologue sequence. In this case parenteral immunization with the peptide containing the critical cross-reactive T cell epitope did not suppress disease. Upon analysis of cytokines produced by the peptide-specific T cells, IL-10 production was found, as was the case with T cells responsive to whole HSP70 protein. Nasal administration of this peptide led to inhibition of subsequent adjuvant arthritis induction (Wendling et al. 2000). Another HSP70 family member, ER chaperone BiP, a 78-kDa glucose regulated protein, was proposed as an autoantigen in RA, as it stimulated proliferative responses of synovium derived T cells from RA patients and not from patients with other joint diseases. Attempts to induce arthritis with BiP in CFA failed, and in line with earlier observations, also this immunization led to resistance against induction of arthritis (Corrigall et al. 2001). Interestingly, more recently, BiP was studied in a first phase clinical trial in RA patients (see below).

A recent analysis of mycobacterial HSP70 was performed in the model of proteoglycan induced arthritis (PGIA) in Balb/c mice. First of all, a mapping exercise was done with mycobacterial HSP70, which revealed the presence of a very conserved T cell epitope at positions 141–155, which we termed the "B29" epitope. When this epitope, in the form of the B29 synthetic peptide, was administered intranasally in PBS (100 ugr) at days -7, -5 and -3 prior to PG immunizations at day 0, a reduced arthritis was noted. And this was also seen in control groups that received whole mycobacterial HSP70 in PBS (30 ugr) and not with control peptide pOVA (100 ugr). Follow-up experiments were then performed with B29 immunization in the presence of DDA as adjuvant, in naïve recipient mice. Splenocytes were sampled 10 days later and from these cells CD4+ CD25+ T cells were collected by cell-sorting and found to be suppressive in a standard suppression assay, using CD3 antibody stimulation. Phenotypic analysis showed upregulated neuropilin (Nrp-1) and LAG-3, both known as markers of regulatory T cells. In addition the percentage of IL-10 positive T cells was also increased. Not surprisingly, a similar phenotypic and functional behavior was noted in these assays with splenocytes from pOVA immunized mice. Interestingly however, when CD4+ CD25+ T cells were transferred into naïve recipient mice prior to induction of PGIA, only the B29 primed T cells protected from disease, whereas the pOVA primed T cells did not. Upon interpretation of all available findings it was concluded that the B29 peptide was inducing a T cell response that cross-reacted with the self-homologs present in the mouse. And indeed, the B29 specific T cell were also showing *in vitro* proliferative responses in the presence of mammalian B29a (mB29a) and B29b (mB29b). Therefore, on the basis of the cross-recognition of endogenously expressed HSP70 self-homologs, the B29 induced Tregs suppressed disease whereas the pOVA induced Tregs did not.

Subsequent studies showed an exceptional tolerance promoting impact of the B29 induced Tregs in the PGIA model. Making use of a congenic marker (CD90.1) present on the transferred Tregs, it was shown that the transferred T cells survived for a long period of time. Even 50 days after transfer, the Tregs were still present in spleen, draining lymph-nodes and the joints. And they were still having the phenotypic qualities of Tregs. Interestingly, when 5 weeks after transfer a depleting CD90.1 monoclonal antibody was infused, the disease suppressive activity of the Tregs was halted. Disease returned in the anti-CD90.1 infused animals and not in

the controls, where apparently the Tregs were still actively engaged in suppression of arthritis. The prevention of disease by early transfer of B29 Tregs was possible with as few as 3.10E5 transferred CD4+ CD25+ T cells. When the T cells were selected on the basis of LAG-3 expression, even the minimal number of 4000 T cells was sufficient to suppress disease (van Herwijnen et al. 2012). Altogether, the B29 mouse experiments have indicated that the targeting of endogenous HSP70 for Treg recognition is a potentially powerful intervention to restore tolerance in inflammatory conditions.

HSP70 in Human Autoimmune Diseases

Analysis of T cell responses to mycobacterial HSP60 in juvenile idiopathic arthritis has shown that such responses were present mainly in cases with self-remitting forms of the disease, namely the subgroup of patients with so-called oligo-articular arthritis and not in the systemic or poly-articular patients. In addition, it was seen that HSP60 specific T cell responses peaked just prior to disease remission. These findings were suggestive of a protective effect of HSP60 specific T cell responses (De Graeff-Meeder et al. 1991, 1995; Prakken et al. 1996). Possibly, similar protective effects can be seen for HSP70. In the case of multiple sclerosis (MS), the presence of HSP70 specific auto-antibodies were found to be a characteristic of patients with relapsing-remitting MS. In patients with primary or secondary progressive MS such antibodies were not seen (Quintana et al. 2008). For BiP comprehensive mapping exercises have been performed with human peripheral blood mononuclear cells (PBMC). In a study by Shoda et al. both effector and regulatory T cell epitopes were identified to be present in BiP (Shoda et al. 2015). The BiP epitope 336–355 was found to induce IL-10 secretion in CD25+ PBMCs obtained from RA patients. In addition, suppression of proliferative T cell responses and pro-inflammatory cytokine production was noted. In mice suppression of disease and induction of Foxp3+ Tregs was obtained following immunization with this epitope.

A recent phase 1 clinical trial with BiP in RA patients showed, besides safety of the approach, some positive clinical effects at the higher doses of peptide intravenously administered (5 and 15 mgr) and not a the 1 mgr dosis. In addition to slight clinical effects, C-reactive protein (CRP), an acute phase protein that acts as a marker of inflammation, was significantly suppressed (Kirkham et al. 2016). Besides induction of Tregs, HSP70 seems to mediate other potentially anti-inflammatory effects. In RA fibroblast-like synoviocytes, the TNF induced production of pro-inflammatory cytokines was inhibited by addition of human HSP70. This effect was claimed to be caused by inhibition of ERK, JNK and p38 MAPK and by inhibitory effects on NF-κB (Luo et al. 2008). Others have shown the HSP70 mediated degradation of the p65 subunit of NF-κB (Tanaka et al. 2014).

Presence of HSP70 Fragments in MHCII in Various Tissues

Elution studies have shown the frequent presence of HSP70 derived protein fragments (peptides) in the groove of MHCII molecules. This can be learned, amongst others, from the SYFPEITHY database of peptides known to bind MHC molecules. In the supplementary data of the Paludan paper (Paludan et al. 2005) it is argued that the cytosol or nucleus derived natural peptides in MHCII are mostly originating from long-lived proteins and their paper shows that among them HSP70 and HSC70 next to GAPDH stand out. And this can be most relevant for the recognition of the HSP70 peptides by Tregs. Tregs are CD4+ T cells that recognize antigens in the context of MHCII. And, as indicated by Shevach, "*it is unlikely that suppression is secondary to the presence of the very small number of autoantigen-specific Treg cells present in the polyclonal population. A more likely scenario is that polyclonal Treg cells are able to control various responses because they are continuously being activated via their TcR by complexes of MHC class II and ubiquitous self-peptides"* (Shevach 2009*)*. It seems that HSP70 is a significant provider of such ubiquitous self-peptides. And as already shown in the case of the B29 peptide of HSP70, the recognizing Tregs can be demonstrated to exist.

A relevant question in this regard is of course how central tolerance with respect to these peptides is organized in the thymus. Following our common understanding of the thymic selection process, it is possible that high affinity T cells will be negatively selected through deletion, whereas intermediate and still relatively high affinities will be leading to the generation of Tregs. A study that explored the MHC-peptide matrix in the human thymus has already revealed the presence of HSP70 fragments in the MHCII molecules of positively selecting thymic epithelial cells (Adamopoulou et al. 2013). And interestingly, a relatively high number of stress response related protein fragments was detected in the thymic DC depleted MHCII positive antigen presenting cell preparations. Also a larger protein fragment was found in the MHC cleft of these cells that included the HSP70-B29 mammalian homolog.

Dengjel et al. have analyzed the MHCII ligandome obtained from nutrient deprived human B cells (Dengjel et al. 2005). The stress caused by nutrient deprivation had led to autophagy, which had influenced the loading of the MHCII compartments of the cell. Possibly through the mechanism of so-called chaperone mediated autophagy (CMA), a chaperone dependent targeting of cytosolic proteins to lysosomes, a preferential loading of MHCII with HSP70 fragments was seen. In the cleft of the HLA-DR4 molecules in this case also our HSP70-B29 was present. Given the known association of HLA-DR4 with RA, this finding is of interest. Apparently, also RA patients with disease predisposing HLA molecules have in principle the genetic capacity to present a proposed disease protective peptide to their T cells. And indeed, with the use of HLA-DR-B29 tetramers the presence of B29 specific Tregs has now been shown in the human T cell repertoire (de Wolf et al. 2016). And very similar to what was seen in mice, the B29 specific T cells were again cross-reactive with the mammalian B29 homologs mB29a and mB29b.

HSP70 Loaded MHCII Is a Treg Inducer

The studies with HSP70-B29 have shown that HSP70 harbors epitopes that can be used to induce disease suppressive anti-inflammatory Tregs (van Herwijnen et al. 2012). And also that these Tregs are functionally active through their cross-recognition of the endogenously expressed and presented mammalian homologs of peptide B29. However, whether or not the endogenous HSP by itself can function as an inducer of Tregs remained unclear. And certainly one may ask the question, whether upregulated HSP at sites of tissue inflammation would be capable of doing so.

In studies by Wieten (Wieten et al. 2010) we have exploited the capacity of a so-called HSP-co-inducer to increase the expression of HSP in the context of cell-stress. This co-inducer was carvacrol, an essential oil obtained from Oregano plant species. When cells, mouse splenocytes or peripheral blood lymphocytes, were exposed to carvacrol for one hour and after that heated (42.5 °C) or exposed to low dose arsenite for two hours and rested in overnight culture, there was a firmly upregulated expression of HSP70 visible with intracellular staining's. When administered intragastrically in mice, carvacrol treated mice showed an upregulated HSP70 in the Peyer's patches, the lymphoid organs lining the gut. Upon analysis, the mesenteric lymphnodes and speen cells appeared to be enriched for Foxp3 positive T cells in carvacrol treated mice and transfer of these cells (selected on the basis of CD3 expression) led to suppression of PGIA in recipient animals. Herewith it seemed that the *in vivo* upregulated HSP70 expression had indeed induced a regulatory T cell population with disease suppressive functionality. When T cell responses against HSP70 were analyzed in carvacrol treated mice, it was found that HSP70 specific T cells had expanded as a consequence of the intragastric carvacrol administration. In more general terms, we have shown herewith that food components can boost the protective cellular response to stress and that the immune system may transduce such information into regulation and suppression of inflammation. What we eat can up-regulate immune regulatory T cells and down-regulate disease.

The induction of Tregs depends most likely on the presence of so-called tolerant DCs (tolDC). Generally, tolDC are characterized by low expression of T cell co-stimulatory molecules, low production of proinflammatory cytokines and high production of immunoregulatory cytokines compared with immunogenic DC (Stoop et al. 2011). It seems that the endogenous upregulation of HSP70 in DCs can turn immunogenic DC into tolerogenic DC. When mouse bone marrow derived DCs (BMDC) was exposed to carvacrol in combination with thermal stress, it turned out that these treated DCs had a reduced capacity to activate pro-inflammatory T cells (Spiering et al. 2012). When such treated DCs were transferred into naïve recipient mice together with CFSE labelled OVA specific T cells, it was found that, of the transferred T cells, especially the Foxp3+ Treg population had expanded. In addition, injection of the carvacrol-thermal stress treated BMDC caused a prophylactic suppression of subsequently induced PGIA.

Altogether, experiments with HSP co-induction have shown that upregulated endogenous HSP70 does trigger an anti-inflammatory Treg cell population. It is

possible that the above mentioned effect of HSP70 on NFκB does contribute to this through the production of tolDC. A specific and irreversible alternative NFκB inhibitor, Bay11–7082 was already tested in a clinical trial using the autologous tolDC as a treatment for RA (Benham et al. 2015). In the same vein, we are preparing grounds for such an autologous tolDC trial, with B29 loaded tolDC. In the latter case, we will follow a recently published protocol of tolDC prepared with exposure of DC to corticosteroids in combination with vitamin D3 (Bell et al. 2017).

Conclusions

Regulatory T cells (Treg) are effector T cells that have the capacity to control inflammation and to maintain herewith the state of self-tolerance. Effective control of inflammation will depend on the cognate interactions with abundantly expressed self-antigens at the site of inflammation. Due to stress imposed on cells exposed to inflammatory mediators HSP are abundantly expressed at sites of inflammation. In the absence of inflammation molecules such as HSP70 can function as default peptide donors for MHCII molecules, which always depend on peptide loading for their cell-surface expression. In this manner, HSP may have a function in the maintenance of self-tolerance.

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