Endogenous stress proteins as targets for anti-inflammatory T cells

The research described in this thesis was carried out at the Department of Infectious Diseases and Immunology, Division of Immunology, Faculty of Veterinary Medicine, Utrecht University, the Netherlands

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Endogenous stress proteins as targets for anti-inflammatory T cells

Endogene stress eiwitten als doelwit voor
anti-inflammatoire T cellen

(met een samenvatting in het Nederlands)

Proefschrift

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CHAPTER 1

General Introduction

Inflammation and tolerance

The mammalian immune system is equipped with a broad array of immune cells and effector responses to protect against invasive pathogenic microorganisms and aberrant tumor growth. Immune responses should be tightly controlled to ensure tolerance to the bodies own cells and proteins (self-antigens) in order to limit damage to the host and to establish or restore immune homeostatic conditions. Immunoregulatory networks comprised of lymphocytes, cytokines, and the tissue itself, coordinate immune responses to pathogen-derived foreign-antigens and to self-antigens (1). B and T cells are important mediators in both effector and regulatory immune responses. These cells of the adaptive arm of the immune system are highly antigen-specific and recognition of their antigen leads to B or T cell effector functioning and long-lasting immunological memory.

Self tolerance

T cells posses an enormous diversity in antigen specificity. By their T cell receptor, T cells can distinguish between different antigens but intrinsically they can not discriminate self and non-self. Through mechanisms of central tolerance in the thymus, T cells that strongly recognize peptides derived from self-proteins undergo apoptosis and are thus deleted from the repertoire (negative selection) (2, 3). This selection is leaky though, and it is nowadays clear that T cells capable of recognizing self-antigens are present in the normal peripheral T cell repertoire (4, 5). To prevent excessive immune responses to self-proteins, their activation is fine-tuned by peripheral tolerance mechanisms. Peripheral tolerance can result from T cell intrinsic tolerance mechanisms; ignorance, when the self-antigen is not encountered in the periphery. Alternatively, tolerance can be induced upon encounter of the antigen in the absence of co-stimulation by anergy, i.e. functional inactivation of the T cell, or activation induced cell death. Besides these intrinsic mechanisms, peripheral tolerance is controlled by regulatory T cells (Treg) (6).

Regulatory T cells

With the capacity to block signature immune responses of various immune cells, like $CD4^+$ and $CD8^+$ T cells, B cells and antigen presenting cells (APC), Treg have a central role in tissue response to injury and inflammation control (7, 8). Nowadays several Treg subsets have been described, to be found in both $CD4^+$ and $CD8^+$ T cell populations (9, 10). Recently, also B cells have been mentioned as modulators of the immune system (11, 12), but these will not be addressed here. $CD4^+$ Treg have been categorized in natural and induced Tregs. Natural Treg express CD4 and CD25. Furthermore, this subset is characterized by constitutive expression of the transcription factor forkhead box P3 (Foxp3). Foxp3 is considered the master regulator gene for Treg development and function (13). Foxp3 signaling leads to up-regulation of Treg associated molecules, like CD25, cytotoxic T

cell associated antigen-4 (CTLA-4) and glucocorticoid-induced TNF receptor family-related gene (GITR) while it represses IL-2, IL-4 and IFN-γ production (13-15). Naturally occurring Treg develop as functionally mature Treg in the thymus and are present from birth. The significance of natural Treg has been clearly demonstrated in athymic nude mice lacking T cells. In these mice, reconstitution with T cells of normal mice depleted of the $CD25^+$ subset yields to multiple autoimmune diseases (16).
Induced Treg, comprised of several subsets, arise in the periphery upon encounter of antigen under tolerogenic conditions. Among those, Tr1 cells induced by repetitive stimulation with antigen in the presence of IL-10. Tr1 do not express Foxp3, low levels of IL-2 and high levels of IL-10. Their inflammation suppressive role has been shown *in vitro*, in T cell mediated colitis models and in transplantation medicine (17, 18). Th3 cells, expressing a $CD4^+CD25^+Foxp3^+$ phenotype, were originally identified upon oral antigen administration and secrete high amounts of TGF-β and variable amounts of IL-10 (19, 20). Another induced Treg subset arises through conversion of naïve $CD4^+CD25^-Foxp3^-$ cells into induced $CD4^+CD25^+Foxp3^+$. This conversion only takes place at low levels of pro-inflammatory cytokines and is IL-2 and TGF-β dependent (21).
For activation Treg require antigen recognition via their T cell receptor. Once activated, Treg can also suppress ongoing immune responses in an antigen independent manner (bystander suppression). Treg can exploit multiple suppressive mechanisms, including the production of anti-inflammatory cytokines, like TGF-β, IL-10 and IL-35, cell contact dependent suppression or killing of effector T cells and conversion of APC into a tolerogenic state (7). By creating a long-lasting regulatory milieu, Treg can promote the outgrowth of new Treg with antigen specificities other than those of the original Treg, designated infectious tolerance. This has been shown in transplantation or type-1 diabetes models where tolerance was maintained even after removal of the eliciting cell population (22, 23). Ultimately enforced Treg suppressive mechanisms will depend on the nature of the immune response, the eliciting agent, the immunological make-up of the host and the site of inflammation as recently summarized (8). Jointly, these mechanisms allow Treg of a single or limited antigen-specificity to control a broad array of immune responses.

Autoimmunity

Despite tight regulation of immune responses towards self proteins, through central and peripheral tolerance mechanisms, loss of self tolerance sometimes occurs. Unwanted and uncontrolled (auto-)aggressive immune responses can lead to autoimmune diseases such as rheumatoid arthritis (RA), type-1 diabetes and multiple sclerosis. Although the target organ and manifested pathology can vary between the different autoimmune diseases, underlying immunological mechanisms have shared characteristics. Most autoimmune diseases arise following a breakdown in the balance between regulatory and auto-aggressive responses (24). The precise aetiology is unknown, but, there is evidence that both environmental and genetic factors add to susceptibility to develop autoimmune

diseases. The pivotal role of Treg in controlling autoimmune responses has been emphasized by the association of defects in Treg regulation and autoimmune disease. These defects can ultimately lead to impaired Treg function or numbers and will be the result of malfunctioning positive or negative selection in the thymus, decreased IL-2 production by effector T cells or defects in IL-10 and TGF-β production by Treg reviewed in (25). In addition, abrogated Treg functioning has been described to result from the action of cytokines present at the site of inflammation (26, 27). Failure of immunoregulatory mechanisms will cause aggressive, pro-inflammatory B and T cell responses to endogenous, normally innocuous, tissue antigens. Subsequently, this will lead to chronic inflammation and tissue damage in the target organs. To date most therapies for autoimmune diseases are substitutive, like administration of insulin to type-1 diabetes patients, or directed towards non specific inhibition of inflammation. For example by blockade of pro-inflammatory cytokines, such as TNF-α and IL-1β or antibody mediated depletion of pathogenic B and T cells (28, 29). Although partially effective, side effects have been observed (30, 31), demonstrating the need for alternative fine-tuned therapies.

Antigen-specific immunotherapy

Immunotherapy with Treg has been explored as therapeutic opportunity to treat a variety of inflammatory diseases. Treg have been found to successfully preserve or restore tolerance in preclinical models for autoimmune diseases, transplantation and allergies. In contrast to conventional general immune suppressive strategies, Treg therapy has the potential of antigen-specificity and can, at least in theory, be tailor made. This would enable highly specific immunomodulation with side effects being limited or absent (32). In practise however, there are key differences between mice and humans and translating therapies from bench to bedside has remained difficult (18, 33, 34). Adoptive transfer of Treg either or not after expansion *in vitro* has been shown to prevent development of inflammatory disease in many autoimmune models but actual suppression of ongoing disease has been shown only in a very limited number of studies (18). An alternative approach is *in vivo* induction of Treg through tolerization with auto-antigens or –peptides derived from the disease inducing antigen. The advantage of peptide-specific therapy will be that it limits the response to disease inciting T cell epitopes.

Mucosal tolerance induction

For *in vivo* tolerance induction, through administration of auto-antigens, the mucosal route is of particular interest. Active non responsiveness is a normal feature at mucosal surfaces as present along the respiratory and gastrointestinal tracts. Several mechanisms ensure mucosal tolerance and prohibit aggravated responses to innocuous antigens encountered at

mucosal surfaces. Exposure to antigens at high dose leads to anergy, through T cell receptor ligation in the in the absence of co-stimulation (35), or to deletion of T cells after CD95 (FAS)-CD95ligand interaction (36). At low dose however, Treg can be induced and these Treg can subsequently exert their regulatory role systemically (37). Controlled induction of mucosal Treg can therefore be powerful means of antigen-specific tolerance induction. Moreover, mucosal administration of antigens would allow easy application with relatively low toxicity risk (38). Oral tolerance to autoantigens, with concurrent suppression of disease, has been successfully established in animal models of autoimmune disease such as rheumatoid arthritis (39, 40), multiple sclerosis (19) and type-1 diabetes (41, 42). and in models of allergy (43) and allograft rejection (44). Clinical trials in humans have been enrolled to induce oral tolerance in autoimmune disease patients (45-47). However, the results were varying, which underlined that translation of successful tolerance induction in animal models to therapeutic intervention in human disease remained difficult.

The choice of antigen for antigen-specific immunotherapy

One of the major pitfalls of immunotherapy based on tolerance induction with disease inducing antigens lies in discrepancies between experimental models and human disease. In animal models peptide-specificity of the disease initiating antigen is known and is usually antigenically related to the tolerogen. In contrast, in human disease the actual disease trigger is frequently elusive and can be multiple. Additionally, through epitope spreading in ongoing inflammation auto-reactive T and B cells can be primed, regardless of the specificity of the initial trigger. This means that specificity of the response can spread from one epitope to another epitope on the same molecule (intramolecular spreading) but also to epitopes on a different molecule (intermolecular spreading) (48). Epitope spreading further complicates tolerance induction to pathogenic antigens or epitopes. It will be challenging therefore, to find alternative auto-antigens as therapeutic agents. Preferably these antigens should be involved in disease perpetuation, yet, independently of the initial trigger. Furthermore, such antigens would meet several characteristics. They should be readily expressed at the site of inflammation, to enable local activation and expansion of Treg but to avoid systemic dampening of host defence systems. Also, to provoke an effective immune response, these antigens should be strongly immunogenic. Interestingly, such self-antigens we may find among highly conserved proteins with homologous sequences found in virtually every cellular organism (see hereunder).

Heat shock proteins

Endowed with the above mentioned characteristics, heat shock proteins (Hsp) have been uncovered as promising candidates for immunotherapy. Hsp are so called stress proteins and are intracellularly expressed in virtually every tissue. Based upon their molecular weight Hsp can be divided into multiple families, including the small Hsp-, Hsp60-, Hsp70- and Hsp90- families (49). Many Hsp have housekeeping functions as chaperones and assist in protein folding and transport. Through these functions, Hsp are imperative for cellular protection against stress, for example by avoiding accumulation of damaged or misfolded proteins during stress (49-51). Expression of some family members is constitutive, however, expression of most Hsp is up-regulated in response to stress (52, 53). This can be virtually every form of stress, for instance high temperature, oxidative stress, nutrient starvation or inflammatory stress. During inflammation increased temperature and pro-inflammatory cytokines can amplify levels of inducible Hsp family members. And enhanced expression of Hsp60, Hsp70 and BiP, an ER restricted Hsp70 family member, at the inflammatory site has been shown in juvenile idiopathic arthritis (JIA) and RA patients (54-57).
The relevance of Hsp for the immune system has been underlined by the finding that after immunization of mice with *Mycobacterium tuberculosis*, 10-20% of the T cells responded to *Mycobacterium Tuberculosis* Hsp60 (58). Similarly, T cell clones obtained from leprosy patients were preferentially reactive to *Mycobacterium leprae* Hsp70 (59). In these studies Hsp were thus uncovered as immunodominant antigens. Because Hsp are abundantly expressed in virtually every organism, the immune system will have to deal with Hsp of both self and foreign origin. Hsp can trigger a broad array of innate and adaptive receptors and signalling pathways, and resulting immune responses can be pro- or anti-inflammatory (60, 61). Hsp has been described to trigger the innate immune system through surface receptors like toll like receptors, CD14, CD40, CD91 and CCR5. Binding of Hsp to these receptors can subsequently modulate phenotype and activation of APC, T and B cells and induce the production of IL-10 (62-66). Furthermore, in the adaptive arm of the immune system T cells that can recognize HS have been observed in healthy individuals (67) and in cord blood (68). Through these innate and adaptive receptors Hsp can exert its immunostimulating and regulatory function (60, 69).

Heat shock proteins and autoimmune disease

As stressed above, Hsp are highly conserved proteins, expressed at the inflammatory site. Hsp are also immunodominant, which combination of qualities would make them attractive auto-antigen candidates for development of antigen-specific immunotherapy. Theoretically, it was possible that Hsp were associated with the cause of autoimmune disease. Since self- and pathogen-derived Hsp are antigenically closely related, activation of auto-aggressive T and B cells, through molecular mimicry, was thought to result in autoimmune disease. However, studies in the adjuvant induced arthritis model in Lewis rats revealed that the

opposite seemed the case. Hsp60 was found as the dominant antigen of disease inducing heat-killed mycobacteria and immunization with Hsp60 before induction of disease protected from development of arthritis (70). The immunomodulatory effects observed with Hsp60 were also found with Hsp70 but not with other highly conserved bacterial proteins (71). Subsequently, the T cell epitopes of Hsp60 were mapped and disease suppressive capacity was found to be mainly restricted to conserved epitopes (72). Then, the immunoregulatory effects were found in various experimental models for inflammatory disease (73-77) and in initial clinical trials in human patients (78, 79). In the animal models, transfer studies showed that T cells mediate protection found upon exogenous Hsp administration (72, 80). Furthermore, in humans, the positive association between T cell responses specific for Hsp60 or conserved Hsp60 peptides and a favourable disease outcome emphasized the immunoregulatory role of Hsp-specific T cell responses (56, 81-83).

Altered heat shock protein expression during ageing and in immune disorders

Stress induced expression is an hallmark of the stress response and its importance has been emphasized by multiple studies showing the positive association between the stress response and longevity reviewed in (84) or with reduced stress-induced tissue damage in mammals summarized in (85-88). Stress-induced Hsp expression has been shown to inversely correlate with age, also in aged immune cells (89-92). Furthermore, Hsp polymorphisms have been associated with inflammatory or autoimmune diseases; like Crohn's disease (93), Alzheimer's disease (94) and pancreatitis (95). Also, altered Hsp expression has been observed in immune disorders (96, 97). In a recent study, in human patients with newly diagnosed type-1 diabetes, decreased stress responses coincided with beta cell directed inflammatory activity (98). Because stress-induced expression is probably important for proper functioning of Hsp-specific T cell regulation, decreased expression of Hsp could cause failure of Hsp immunoregulation. Thus, although many factors are involved, failing stress responses might contribute to the enhanced risk of developing immune disorders at increasing age. Boosting of Hsp inducibility may then restore immune homeostasis (Figure 1).

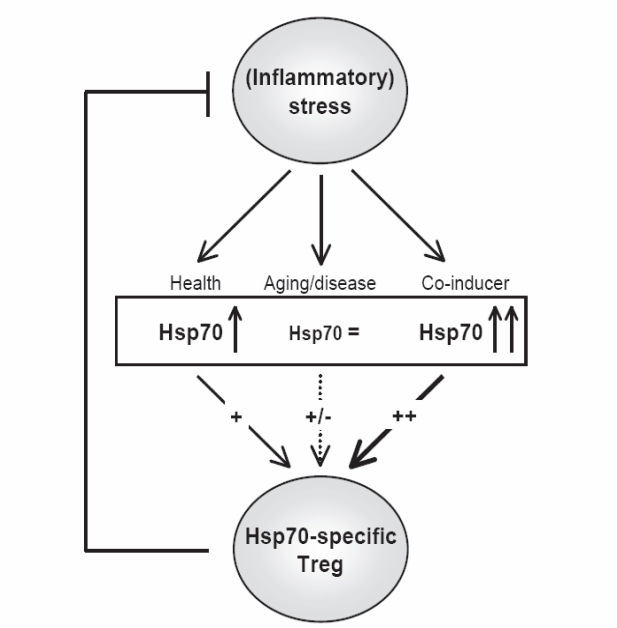

Figure 1. Influence of stress-induced Hsp70 expression on Hsp70-specific immunoregulation.
During inflammation, enhanced Hsp70 levels will amplify immunoregulation by Hsp70-specific anti-inflammatory T cells. Upon activation, these T cells can dampen ongoing inflammation. Decreased induction of Hsp70 upon stress has been associated with aging and several immune disorders and may cause failure of induction and or activation of Hsp70-specific anti-inflammatory T cells. Lack of regulation will increase the risk for development of chronic inflammatory disease. Boosting Hsp70 stress-inducibility with co-inducers of the stress response could restore or enhance Hsp-specific immunoregulation.

Aim of the thesis

Breakdown of immunoregulatory mechanisms can cause loss of self tolerance and chronic inflammatory disease. Antigen-specific targeting of Treg with self-antigens that participate in inflammation irrespective of the disease would ideally allow site-specific, tailor made intervention in autoimmune disease. Hsp possess several features that make them promising candidate antigens; Hsp, are highly conserved among species, immunodominant and their expression is up-regulated by cells in the inflamed tissue. The regulatory potential of Hsp and Hsp-specific T cell responses has been emphasized by suppression of inflammatory disease upon exogenous Hsp administration in various experimental models. Also in patients the immunomodulatory potential of Hsp has been observed. Enhanced Hsp expression can be seen in virtually every inflammatory condition and would lead to local activation of Hsp-specific Treg that can dampen ongoing inflammation. Therefore, enhanced levels of endogenous Hsp could be targets for anti-inflammatory Treg and in that way provide an inhibitory feedback mechanism.
Among the various Hsp, particularly Hsp70 is highly stress inducible. But stress inducibility of Hsp70 is decreased at increasing age. Therefore, we postulate that when expression of the self-antigen is reduced, Hsp mediated immunoregulation fails. Various studies have shown that Hsp70 inducibility can be enhanced or restored with drugs and that this augments cellular resistance to stress. It is yet not known if the immune system can actually respond to boosted expression of endogenous Hsp.

Basic knowledge on the regulatory mechanisms of Hsp-specific Treg in general and of Hsp70 in particular will be indispensable to enlarge and refine current knowledge on Hsp as therapeutic agents. Additionally, it might lead to development of novel strategies to boost immune fitness by Hsp mediated tolerance induction. Therefore, the aim of the studies described in this thesis is twofold. First, we want to further unravel the mechanism of Hsp70-specific T cell regulation. Second, we address the potential of Hsp70 as therapeutic agent, on the one hand by antigen- or epitope-specific immunotherapy with exogenous Hsp70, while on the other hand via a thus far unexplored strategy; by compound-induced up-regulation of endogenous Hsp70.

Outline of the thesis

In **chapter 2** we discuss Hsp-mediated immunoregulation and put forward how Hsp inducing compounds can be exploited to boost anti-inflammatory T cells. We also address the state of the art in the field of drug-induced up-regulation of Hsp. To enable mechanistic studies on the effect of boosted Hsp expression on Hsp immunoregulation, in **chapter 3** *in vitro* systems to monitor Hsp70 expression and induction by Hsp inducing compounds are investigated. In **Chapter 4** the Hsp70-specific T cell response is boosted by Hsp70 immunization and the outcome is studied in the proteoglycan-induced arthritis model (PGIA). Furthermore, the role of the regulatory cytokine IL-10 is addressed. Since the mucosal route can be an attractive way for administration of antigens, peptides or drugs, we consider mechanisms of mucosal tolerance induction in the PGIA model in **chapter 5**, by analysis of Treg induction and functioning upon oral or nasal proteoglycan administration. In **chapter 6** the effect of nasal treatment with Hsp70 is examined. Furthermore, we set out to identify immunodominant T cell epitopes of Hsp70 in BALB/c mice and to characterize T cell responses and regulatory capacity of dominant peptides in PGIA. In addition, we study loading and presentation of the peptides on both mouse and human MHC class II and the clinical relevance of the identified peptides for epitope-specific immunotherapy means. In **chapter 7** one of the co-inducers found in chapter 3 is studied in more detail by addressing how the food component carvacrol amplifies Hsp70-specific protective T cell responses. In addition the effect of enhanced endogenous stress protein expression on T cell regulation in the PGIA model is described. In **chapter 8** a general discussion is presented on the impact of novel findings by us and others on Hsp as therapeutic agents.

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CHAPTER 2

Cell stress-induced Hsp are targets of regulatory T cells:

a role for Hsp-inducing compounds as anti-inflammatory immunomodulators?

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Abstract

T cell responses to heat shock proteins (Hsp) have disease suppressive activities through production of anti-inflammatory cytokines in patients and in models of inflammatory diseases. There is evidence that the anti-inflammatory activity of Hsp specific T cells depends on their recognition of endogenous Hsp epitopes as expressed by stressed cells at sites of inflammation. Previously we have demonstrated that such T cells can be induced by conserved sequences of microbial Hsp. Now we propose that drug induced up-regulation of endogenous Hsp can contribute to anti-inflammatory T cell regulation.

Cell stress leads to Hsp up-regulation

Heat Shock Protein (Hsp) expression can be induced in cells through various forms of stress, such as hyperthermia (fever), nutrient starvation, oxidative- and toxic stress and exposure to inflammatory cytokines (1, 2) On the basis of their molecular weights, Hsp can be organized in Hsp families, including the Hsp100, Hsp90, Hsp70, Hsp60, Hsp40 and the small Heat Shock Protein families (3). Most likely because of their critical significance for cell survival under conditions of stress, the primary structures of many individual members of the Hsp families remained highly conserved throughout evolution. For some Hsp family members expression is mainly constitutive and for others the expression is highly inducible. Depending on the cell type and applied stress, protein expression of inducible Hsp family members is increased. The inducible Hsp70 family members are among the most prominent proteins up-regulated under stress. In addition, the expression of HSC70 is an example of constitutive Hsp expression (1, 4, 5).

Upon stress, augmented expression of Hsp is controlled by a family of Heat Shock transcription Factors (HSFs) among which HSF1 is essential for induction of the so-called heat shock response (HSR). Under physiologic conditions HSF1 is maintained in its monomeric, inactive form by binding to molecular chaperones like Hsp70 and Hsp90. After stress, HSF1 is released and translocates to the nucleus. Subsequently, trimerization, binding to Heat Shock Elements (HSE) and hyperphosphorylation finally result in transcription of Hsp (6, 7). Since altered Hsp function has been associated with the development of several diseases, including immune dysfunctions (8), compound induced modulation of Hsp expression became an emerging field of drug development and will be discussed briefly below. We will focus on compounds that can be administered orally, since the intestinal mucosa seems most relevant for induction of T cell immunoregulation. In addition, up-regulation of Hsp by hyperthermia will be discussed.

The enhanced Hsp induction through compounds which we discuss can obviously be of relevance for aging, as elderly individuals have a known reduced Hsp inducibility (9, 10). In this way such artificial Hsp enhancement could counter the age dependent increased risk of autoimmune diseases, which is a possible reflection of the growing dysbalances of the immune system during senescence.

Several drug related compounds are Hsp expression enhancers

Activators of HSF can be categorized into Hsp inducers and co-inducers. An inducer activates HSF in the absence of additional stress. A co-inducer partially activates components of the HSR, further stress signals are required for full activation of Hsp transcription (11, 12).

Compounds that inhibit Hsp90 can act as Hsp inducers. Hsp90 inhibitors, like the benzoquinone ansamycins herbamycin A and geldanamycin, have been shown to bind to Hsp90 ensuing in a disturbance in the binding of Hsp90 to HSF. Then, released HSF is further activated leading to Hsp expression (13, 14).

Non-steroidal anti-inflammatory drugs (NSAIDS) can co-induce Hsp expression. Sodium salicylate induces trimerization and DNA binding of HSF without induction of hyperphosphorylation (15). Indomethacin, in contrast to Sodium Salycilate, can induce both DNA binding and hyperphosporylation (16). In the search for non toxic Hsp inducing compounds, ample attention was paid to herbal medicines. Several of them are known for their anti-inflammatory and anti-tumour effects. Celastrol, a member of the triterpene family of compounds, has been shown to activate HSF1 with kinetics similar to that of heat stress. Moreover, Celastrol can amplify heat shock induced Hsp expression (17). The herbal medicine constituents, glycyrrhizin and paeoniflorin, were reported to have an enhancing effect on Hsp expression. Glycyrrhizin was acting as a co-inducer. In contrast, Paeoniflorin could induce Hsp expression without supplementary stress (18).
Curcumin, the major compound of the seasoning tumeric, was described to increase Hsp27, αB crystallin and Hsp70 expression in combination with arsenite or heat stress. In contrast, treatment with curcumin alone did not have an effect on Hsp expression. The elevated Hsp expression was seen in both cultured cells and after *in vivo* administration, in rat tissues (19). The hydroxyl amine derivates, arimoclomol and bimoclomol have been suggested to co-induce Hsp expression. Arimoclomol induces Hsp70 and Hsp90 expression (20). Bimoclomol has been reported, to have cytoprotective effects by up-regulation of Hsps in several *in vivo* and *in vitro* models (21-23). Anti ulcer drugs like geranyl geranyl acetone (GGA), rebamipide and carbenoxolone can induce or co-induce Hsps. Among the anti-ulcer drugs GGA, an acrylic isoprenoid, is best described. Oral treatment with GGA protected from ischemia reperfusion induced liver injury via Hsp up regulation (24). Depending on the cell type GGA can either act as an inducer or a co-inducer. Recently, GGA has been described to bind to Hsp70 leading to dissociation of Hsp70 from HSF after which free HSF could bind to DNA (25).

Hyperthermia induced Hsp up-regulation

Hyperthermia pre-treatment can induce thermo-tolerance and has been shown to be protective against various types of injury. Hsp up-regulation upon heat shock is extensively studied in multiple cell lines, primary cell cultures and *in vivo* models. Hsp up-regulation and the kinetics thereof seem to depend very much on cell type and heat shock conditions used.
In conventional models for *in vivo* hyperthermia, a short heat treatment, 15-20 minutes, is applied at relatively high temperature (42-43°C). Heat preconditioning of rats at 42°C for 15 minutes, attenuates acute ischemic renal injury. In the latter study, protein expression of Hsp70 but not Hsp27, Hsp32 and Hsp90 was significantly increased. Administration of quercitin, an Hsp70 inhibitor, almost completely reversed protection (26). Ostberg *et al.* propose that fever-range whole body hyperthermia (FR-WBH, 39.5-40°C for 6h) is a more accurate model for heat stress than the conventional models because it more closely mimics physiological conditions. In this model up regulation of Hsp70 and Hsp110 was found in

multiple mouse tissues (27). Likewise, we could detect increased Hsp70 but not Hsp60 expression after 6h *in vivo* FR-WBH in spleen cells of mice (chapter 3 this thesis).

Hsp inducing compounds can be anti-inflammatory by inhibiting NF-κB

Many compounds that (co-)induce the heat shock response (HSR) are also NF-κB inhibitors. In the non-inflammatory state NF-κB is kept inactive in its cytosolic form complexed to its inhibitor I-κBα. Firstly, proteasome inhibitors prevent degradation of I-κBα by the proteasome and thereby prevent activation of NF-κB and its translocation to the nucleus. At the same time proteasome inhibition increases the cytosolic content of incorrectly folded proteins, thereby inducing the HSR.

Secondly, many inducers of the HSR are known to block the NF-κB pathway directly not only by stabilizing I-κBα but also by inducing I-κBα gene expression (28). Therefore, various compounds may act in an anti-inflammatory fashion directly in the cell by blocking the pro-inflammatory NF-κB cascade, but simultaneously through the induction of the HSR thereby evoking immune modulation by the provision of targets for Hsp specific regulatory T cells.

Endogenous cell stress induced Hsp at the site of inflammation are targets of regulatory T cells

The role of heat shock proteins in experimental models of inflammatory diseases was first discovered in the model of adjuvant induced arthritis in rats (29). Disease associated T cell lines and clones raised against heat-killed mycobacteria, the disease triggering antigen in this model, were found to recognize a protein cloned from *M. bovis* BCG with a molecular weight of 65kDa. This 65kDa protein turned out to be the Hsp60 of mycobacteria. Interestingly, immunization studies with this recombinant protein in the same rat arthritis model showed that Hsp60 immunized animals developed a resistance against the induction of adjuvant arthritis.

Steve Anderton (30) was able to generate a series of T cell lines from rats after immunization with mycobacterial Hsp60. These T cells were tested in adoptive transfer experiments and one of them was transferring resistance. This particular T cell line was found to recognize a highly conserved sequence of Hsp60 and as expected, this T cell did cross-recognize the mammalian homologue of the mycobacterial protein: rat Hsp60. The other T cells that did not transfer resistance were also mapped and none of them cross-recognized mammalian Hsp60.

The same was also found for mycobacterial Hsp70 (31). Immunization with mycobacterial Hsp70 protected against adjuvant arthritis in rats, and also protected in a model induced with a synthetic oily compound called avridine. Also here mapping studies revealed that only T cells recognizing highly conserved sequences were transferring protection and were cross-responding to the mammalian homologous proteins. Various studies using different routes of exposure to peptides representing conserved Hsp60 and Hsp70 T cell epitopes,

have shown immunoregulatory effects of these conserved sequences in a multitude of disease models.

From these studies we must conclude that immune exposure to microbial Hsp was capable of inducing a regulatory T cell response and that such regulation depended on a T cell response that included a repertoire of (endogenous) self Hsp specific T cells.

The existence of self-Hsp reactive T cells has been demonstrated in many different studies, even in human umbilical cord lymphocytes (32) and in mice transgenic for Hsp60 (33). Apparently thymus selection allows such a repertoire to develop. Microbial Hsp are dominantly immunogenic and frequencies of Hsp-specific T cells can be very high following microbial exposure. It makes sense to suppose that this T cell stems from a repertoire that has been selected in the thymus by self (endogenous) Hsp peptides. Low affinity interactions for such self Hsp must have stimulated positive selection and must have allowed the self Hsp reactive T cells to survive negative selection. In the peripheral immune system, when the cells have left the central lymphoid organs, microbial Hsp can be the full agonists for these T cells compatible with the immunodominant character of these Hsp. At the same time, when self Hsp is expressed in the periphery, under conditions of cell stress, the self Hsp can act as a partial agonist producing a regulated or actively regulatory response in these T cells .

In addition, at the site of inflammation Hsp will be up-regulated in all (stressed) cells of which the majority will be tissue cells lacking co-stimulatory molecules. In the absence of co-stimulatory molecules T cells will adopt a state of anergy or regulation (34). Through these mechanisms Hsp specific T cells can adopt a regulatory phenotype upon antigen recognition in the periphery of the immune system. And this may explain the tolerant state of these cells and that they can be present at high frequencies without causing damage. It is possible that this tendency of these cells to stay in a tolerant or regulatory state is further promoted by mucosal tolerance in the gut associated lymphoid system (GALT) for abundantly present microbial Hsp from the gut flora. The tolerance for this collection of Hsp in the gut microbiota will be dominated by tolerance for the conserved sequences, as these are shared among the variety of bacterial species present. Thus, there may be a natural focus on the conserved sequences of Hsp to drive a repertoire of regulatory T cells.

In various studies Hsp reactive T cells have been seen to produce the immuno-regulatory cytokine IL10 (35-37). These cells are most likely regulatory T cells, induced in the periphery and reflecting mechanisms known to contribute to development of peripheral tolerance.

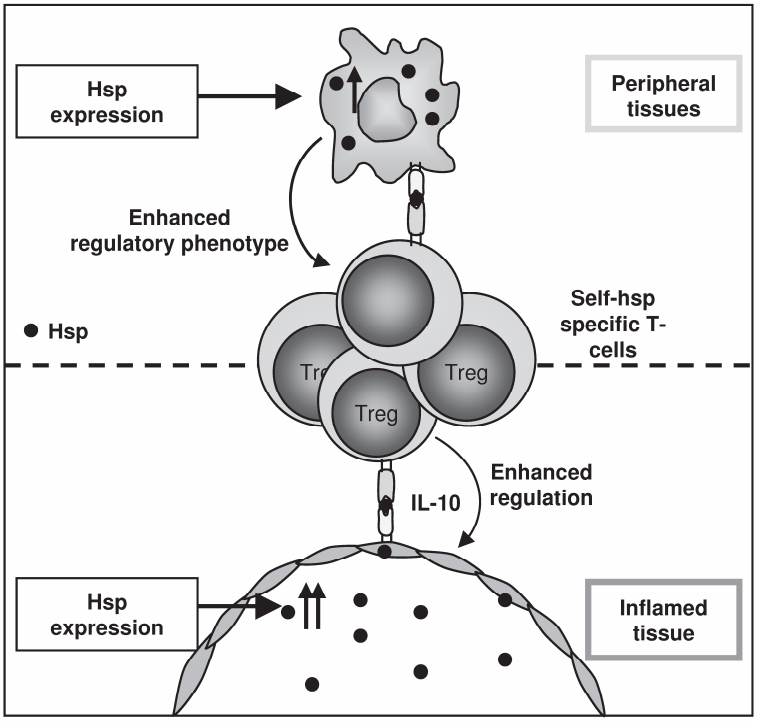
Some studies have also suggested that Hsp are antigens seen by natural Tregs (38). Along the same line, studies in children with juvenile idiopathic arthritis (JIA) and adults with rheumatoid arthritis (RA) have indicated the potential of Hsp to trigger in T cells the presence of FoxP3 which is a marker for natural Tregs.

Thus, given the fact that regulatory T cells induced by microbial Hsp have been seen to cross-recognize endogenous Hsp through their specificity for conserved Hsp sequences, it seems that up-regulated endogenous Hsp in stressed cells is the target and the possible initiator of the local regulatory activity of these T cells.

How levels of endogenous expression of Hsp at sites of inflammation or elsewhere in the body would influence that capacity of endogenous Hsp reactive T cells to maintain or display regulatory activity is unknown.

Experimental manipulation of endogenous Hsp expression, during disease or during maturation of the immune system may well lead to a further understanding of the possible contribution of cellular Hsp expression to T cell mediated immune regulation.

Some drug compounds are known to have effects on endogenous Hsp levels as discussed above. In the following section we will discuss some effects of such compounds in disease models with T cell involvement.



Legend to figure

Hsp inducing and co-inducing compounds enhance the expression of Hsp in tissue cells, including MHCII positive cells which then present Hsp epitopes to Hsp specific CD4+ T cells. A variety of mechanisms (39) (microbial Hsp exposure in the tolerising gut mucosa; presentation by non-professional APC lacking co-stimulatory molecules; Hsp acting as partial agonist; priming in the presence of stress cytokines such as IL10) are imposing a regulatory phenotype on these CD4+ T cells. These Hsp specific regulatory T cells can recognize induced Hsp as presented by stressed cells at the site of inflammation and dampen inflammation by production of suppressive cytokines such as IL10. And also here the Hsp inducing compounds can further enhance local Hsp expression.

Administration of Hsp up-regulators and effects on T cells in chronic inflammatory diseases

Earlier we described that T cells can respond to endogenous Hsp and that this can lead to an anti-inflammatory phenotype. In other words, Hsp up-regulated in inflamed tissues can be targets for Hsp specific regulatory T cells (39). A relative deficiency of this protective activity in chronic inflammatory diseases can be due to a reduction of the Hsp reactive T cell population itself or to a relatively low expression of Hsp in the inflamed tissue where Hsp are required as targets for triggering the regulatory T cells locally. Elsewhere we have reviewed how administration of (immunization with) Hsp can reinforce the regulatory Hsp specific T cells (39). Here we will present examples from the literature in which (co-)inducers of Hsp may have enhanced the expression of Hsp to serve as better targets for the Hsp specific regulatory T cells capable to inhibit T cell mediated chronic inflammatory diseases. Additionally, enhanced expression in non-inflamed tissues also may have reinforced the induction of Hsp specific regulatory T cells.

Geranylgeranylacetone (GGA): GGA, a specific Hsp (co-)inducer, is given to patients for anti-ulcer therapy, its mode of action being local gastric parietal cell protection. In the experimental model of autoimmune uveoretinitis (EAU) in mice GGA treatment reduced disease which was associated with suppressed T cell responses against the disease inducing antigen, IRBP peptide 1-20 (40). GGA was also found to be protective in trinitrobenzene sulfonic acid-induced (TNBS) colitis (41), a T cell mediated model for colitis and in dextran sulfate sodium-induced (DSS) colitis in mice (42).

Curcumin: Curcumin is a biologically active component of turmeric, inhibits NF-κB and is known to be a co-inducer of stress proteins (19). Recently, a turmeric extract containing curcumin, given orally was shown to inhibit joint inflammation and periarticular joint destruction in a dose-dependent manner in the T cell mediated, experimental model of streptococcal cell wall-induced arthritis (43). Also, an anti-arthritic effect of curcumin has been reported in one small clinical study of rheumatoid arthritis (RA) and in other studies of arthritis in animals (44, 45). In other patient studies oral curcumin was found to be a safe medication for maintaining remission in quiescent ulcerative colitis (46) and it induced improvements in other forms of inflammatory bowel disease (47). Recently, it has been suggested that curcumin's reported beneficial effects in arthritis and colitis, but also in other T cell mediated chronic inflammatory disorders like allergy, asthma, atherosclerosis and diabetes, might be due in part to its ability to modulate the immune system, and that these findings warrant further consideration of curcumin as a therapy for immune disorders (48).

Geldanamycin: Geldanamycin by its direct binding to Hsp90 activates the HSR response and induces Hsp, and at the same time, inhibits NF-κB by inducing selective degradation (through autophagy) of the IkappaB kinase (IKK). IKK normally phosphorylates I-κB, which then detaches from cytosolic NF-κB enabling translocation to the nucleus of the latter (49).

Administration of 17-allylamino-17-demethoxygeldanamycin (17-AAG), a less toxic derivative of the naturally occurring geldanamycin, in mice immunized with myelin oligodendrocyte glycoprotein peptide to induce experimental autoimmune encephalomyelitis (EAE), prevented disease when given at an early time, and reduced clinical symptoms when given during ongoing disease. T cells from treated mice showed a reduced response to immunogen re-stimulation (50).

Hyperthermia
In EAE whole body hyperthermia (WBH) has been described to strongly reduce the incidence and severity of EAE (51). T-cell activation, assessed by the production of interferon gamma (IFN-γ) in response to the EAE inducing immunogen myeloid oligodendrocyte antigen (35-55), was also decreased by the HSR.

Thus, in several disease models endogenous Hsp induction has been seen to lead to suppression of disease. Although mechanisms involved could be manifold, in some cases evidence for immuno-modulatory effects was already obtained.

Hsp induction and the possible contribution of mucosal tolerance to immuno-regulation
Most Hsp inducers are administered orally. Therefore, we have to consider the effects of these drugs in inducing local Hsp up-regulation in the mucosal tissues.
The mucosal immune system, as present for example in the gastrointestinal tract and airways, guards the major entry sites of the body against foreign antigens, and is thereto especially equipped with the ability to adjust the outcome of the immune response depending on the type of antigen encountered. The specific down regulation of unwanted and unnecessary systemic immune responses to innocuous antigens upon mucosal antigen encounter is called mucosal tolerance and has been described for both the intestinal and the respiratory mucosa (52, 53). This systemic immunological hypo-responsiveness is characterized by a reduced T and/or B cell response to the antigen firstly applied at the mucosal surface. A major mechanism contributing to mucosal tolerance is the active induction of antigen specific regulatory T cells, characterized by the secretion of regulatory cytokines such as IL-10 (53, 54).
Mucosal application of bacterial Hsps has been shown to be a potent means of regulatory T cell induction in several models of chronic inflammatory diseases such as atherosclerosis, arthritis and diabetes (31, 55). Since up-regulation of self-Hsps is part of the inflammatory response in these diseases, the induction of self-Hsp-cross reactive T cells upon mucosal application of the highly homologous bacterial Hsp might seem an attractive explanation for the development of the mucosally induced regulatory T cells with specificity for otherwise very immunogenic and potentially pro-inflammatory bacterial proteins. Indeed, in several studies of the protective mechanism of Hsp, self-Hsp cross-reactive T cells were found to

produce regulatory cytokines, such as IL-10 and exhibit regulatory activity (Wieten et al in prep, (31, 56)). In addition, mycobacterial Hsp70 mediated protection in the experimental mouse model of proteoglycan induced arthritis not only induced IL10 producing Hsp-specific T cells, but also proteoglycan-specific T cells that produced IL-10 upon antigen specific stimulation (Wieten et al unpublished results). This indicated that bacterial-Hsp application induced a regulatory phenotype in both Hsp specific and disease associated PG-specific T cells. These data entail that Hsps might be a therapeutic target for the induction of mucosal tolerance in cases where the auto-antigen is unknown and that microbial Hsp can induce antigen specific regulatory T cells.

Various mechanisms have been described to be important in the suppressive function of regulatory T cells. In the case of Hsp-specific regulatory T cells it is attractive to speculate that these cells play an important role at the site of inflammation where self-Hsp is abundantly present. Mucosal Hsp-specific regulatory T cells can dampen the ongoing inflammatory response by local production of IL-10 at the site of inflammation thereby down-regulating pro-inflammatory cells and cytokines. In addition, mucosal regulatory T cells have been shown to use infectious tolerance as a mechanism to expand their regulatory function and convert naïve T cells to differentiate into regulatory T cells (57). Such a mechanism might explain the observed IL-10 production by arthritogenic T cells in the proteoglycan induced arthritis model and might expand the regulation to different cells or antigens involved in the inflammatory response. Moreover, these mechanisms might also play a role during the dampening of the inflammatory response under normal conditions thereby preventing excessive inflammation and subsequent organ damage.

For the reasons given above, the exposure to (microbial) Hsp at the mucosal surface of the gut, may be a major factor in endowing the repertoire of Hsp specific T cells with regulatory potential. A local action of our Hsp inducing compounds at the gut mucosa, in up-regulating endogenous self-Hsp in the mucosa may therefore prepare the mucosa for attracting the attention of Hsp specific T cells and for creating a local environment optimal for setting these T cells into a regulatory mode.

Clinical development of Hsp mediated immune intervention in chronic inflammatory diseases

Studies in juvenile idiopathic arthritis (JIA) have shown that T cell responses to human Hsp60 are predominantly found in patients with remitting forms of this disease (oligo-articular). In addition remission was shown to be preceded by raised proliferative responses to human Hsp60. In a recent study Kamphuis et al. (37) have shown that such patients do respond to conserved Hsp60 sequences and that the T cells of such patients do produce high levels of IL10.

First human trials have confirmed the immuno-modulatory capacity of Hsp-derived peptides in chronic autoimmunity. In type 1 diabetes a mammalian Hsp60 peptide –diaPep277- encomprizing a known T cell epitope in mice and humans has been used to immunize

subcutaneously. This procedure was seen to temporarily arrest inflammation and beta-cell destruction in the pancreas resulting in a decreased insulin dependency. In the peptide specific T cells the inflammatory cytokine phenotype was seen to shift into the development of a regulatory (IL10 and IL4) cytokine phenotype (37, 58, 59).
The therapeutic potential of mucosal delivery of Hsp peptides was indicated by a study in RA, where a DnaJ (Hsp70 complex) peptide was given orally. Also here a shift towards a regulatory cytokine profile was noted in the peptide specific T cells. In a recent follow-up phase II trial a favorable clinical response was seen in the peptide treated group especially when the peptide was given in combination with hydroxychoroquine, a second line anti-rheumatic drug known to improve antigen presentation (Albani et al., personal comm.). Although in their infancy, based on first results, these Hsp directed antigen specific approaches are promising and may lead to future effective therapies for the control of a wide spectrum of inflammatory diseases. If so, an important issue to address will be whether or not such interventions can lead to permanent remissions of disease. It may well be possible, that the result of such interventions will be determined, amongst others, by the continued exposure of the immune system to the relevant antigens, Hsp in this case. As long as we are not aware of the exact factors that have led to the induction of disease or that have prepared the ground for disease producing immune deregulation, we must entertain the possibility that promotion of stress inducibility of endogenous Hsp will be essential for the (permanent) establishment of disease suppressing immune regulation. The use of oral Hsp up-regulating compounds for this purpose is then an attractive and further to be explored possibility.

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CHAPTER 3

Hsp70 expression and induction as a readout for detection of immune modulatory components in food

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Abstract

Stress proteins such as heat shock proteins (Hsp) are up-regulated in cells in response to various forms of stress, like thermal and oxidative stress and inflammation. Hsp prevent cellular damage and increase immunoregulation by the activation of anti-inflammatory T cells. Decreased capacity for stress-induced Hsp expression is associated with immune disorders. Thus, therapeutic boosting Hsp expression might restore or enhance cellular stress resistance and immunoregulation. Especially food- or herb-derived phytonutrients may be attractive compounds to restore optimal Hsp expression in response to stress. In the present study we explored three read-out systems to monitor Hsp70 expression in manner relevant for the immune system and evaluated novel Hsp co-inducers. First, intracellular staining and analysis by flow cytometry was used to detect stress and/or dietary compound-induced Hsp70 expression in multiple rodent cell types efficiently. This system was used to screen a panel of food-derived extracts with potent anti-oxidant capacity. This strategy yielded the identity of several new enhancers of stress-induced Hsp70 expression, amongst them carvacrol, found in thyme and oregano. Second, $CD4^+$ T cell hybridomas were generated that specifically recognized an immunodominant Hsp70 peptide. These hybridomas were used to show that carvacrol enhanced Hsp70 levels increased T cell activation. Third, we generated a DNAJB1-luc-O23 reporter cell line to show that carvacrol increased the transcriptional activation of a heat shock promoter in the presence of arsenite. These assay systems are generally applicable to identify compounds that affect the Hsp level in cells of the immune system.

Introduction

Heat shock proteins (Hsp) are intracellular proteins important for maintenance of cellular and immune homeostasis (1). Through their chaperone and refolding capacity Hsp assist in transport and refolding of damaged proteins, processes that are essential to maintain cellular integrity during stress (2, 3). Hsp are up-regulated in response to various forms of stress, like oxidative-, heat- and inflammatory-stress (4). Based on their molecular weight, Hsp are divided into multiple families and expression of family members can be either constitutive or stress inducible. The Hsp70 family consists of 13 members among which HspA1A and HspA1B (here further referred to as Hsp70) are the best known inducible Hsp70 family members. Also, some members of the DNAJ (Hsp40) and Hsp90 families are highly stress inducible (5, 6). Transcription of inducible Hsp is initiated by binding of heat shock factor 1 (HSF1) to heat shock binding elements (HSE) in the promoter region (7).
The expression of inducible Hsp family members is increased in cells in inflamed tissue. For example, in patients with rheumatoid arthritis or juvenile idiopathic arthritis, augmented Hsp expression was observed in the inflamed synovium and found to be important for proper functioning of Hsp-specific regulatory T cells (8-10). The immunoregulatory potential of stress proteins is becoming increasingly clear (11-13). In experimental models of inflammatory disease, like arthritis, diabetes and atherosclerosis, administration of exogenous Hsp or Hsp-peptides suppressed disease via the induction of T cells that specifically recognized Hsp60 or Hsp70 (for review, see (14)). Also in recent clinical trials in patients with inflammatory disease, administration of Hsp-derived peptides has been proven successful (15, 16).

Decreasing stress inducibility of Hsp with increasing age has been reported (17, 18). Furthermore, altered Hsp expression has been associated with immune dysfunctions (19, 20). An attenuated stress response, as observed during aging, might lead to failure of Hsp-specific T cell regulation and add to an increased susceptibility to develop inflammatory disease in the aged individual. To enhance cellular fitness and stress resistance several compounds of different origins have been tested for their capacity to induce or co-induce Hsp expression (21-23). Beneficial effects of compound-induced stress protein expression on the outcome of experimental diseases such as cardiac dysfunction and neurodegenerative disease have been observed in many models (24-28). However, the outcome of boosting the stress response on Hsp-specific T cell regulation has not been studied before. Boosting Hsp expression with Hsp co-inducers could restore or enhance physiological immunoregulation. In particular, food- or herb-derived agents may enable safe and non-toxic promotion of the heat shock response and probably the Hsp-specific T cell response. In most studies that screen for potential Hsp-inducing compounds, Hsp70 expression was used as a readout. From the T cell perspective, however, enhanced expression of Hsp will only have an effect if this coincides with augmented Hsp-peptide

presentation on the MHC of antigen presenting cells (APC). To study the outcome of manipulation of the stress response in a way that is also relevant for the immune system, additional screening methods are required. In the present study we explored three different techniques to monitor Hsp70 expression. We show that analysis of intracellular Hsp70 by flow cytometry allows fast and cell-specific analysis of changed Hsp levels. Additionally, we generated Hsp70-specific T cell hybridomas to detect Hsp peptide presentation on MHC class II by APC and we generated a heat shock promoter driven luciferase reporter system. Using three techniques we detected novel compounds that enhance expression of Hsp.

Materials and Methods

Cell-culture

RAW264.7 and A20 cell-lines were purchased from American Type Culture Collection. CTLL-16 (29) was obtained from Sanquin Blood Bank. A20 and CTLL-16 were routinely cultured in Optimem supplemented with 5% FBS (Bodinco B.V.). RAW264.7 cells were cultured in DMEM supplemented with 5% FBS and 100 units ml^{-1} penicillin and 100 µg ml^{-1} streptomycin (p/s) (Gibco BRL). m-IC_{c12} intestinal cells, primary spleen cells and bone marrow derived dendritic cells (BMDC) were cultured in IMDM with 10% FBS and p/s. For spleen and BMDC cultures, $5x10^{-5}$ M 2-mercaptoethanol was added to the culture medium. BMDC were isolated from the bone marrow of 9-12 week old BALB/c mice and cultured for 7 days in the presence of 10 ng ml^{-1} GM-CSF (Cytogen) according to (30) with minor modifications. BMDC were used on day 8 for *in vitro* assays. All cultures were maintained in a humidified 5% CO_2 atmosphere at 37°C.

Chemicals

Geldanamycin was obtained from Invivogen. Curcumin and carvacrol were obtained from Sigma. Food-derived extracts were prepared as described previously (31, 32). In brief, hydrophilic fractions of samples from fruit and vegetables were obtained extracting a homogenized sample with water under agitation for 15 min at room temperature. Supernatant was collected after 10 min of centrifugation at 1000xg. To obtain the lipophilic fraction, the pulp residue was reextracted with acetone as described for hydrophilic fraction. Black tea extract was prepared by brewing an infusion (~2 g) in 250 ml boiling water for 5 min. To obtain chocolate extracts, chocolate was defatted with n-hexane for 5 min in an ultrasonic bath at 30°C after which the suspension was centrifuged for 10 min at 1000xg. Subsequently, the pellet was extracted for 10 min under agitation with a mixture of acetone/water (70:30) in an ultrasonic bath at 30°C. After 10 min of centrifugation at 1000xg supernatant was collected.

In vitro heat treatment and exposure to compounds

For *in vitro* heat shock treatment cells were seeded in 24 or 48 wells plates at 1-2 ml per well ($2x10^6$ cells ml^{-1}). Then, cells were placed overnight in an incubator at 37°C and 5% CO_2 atmosphere. Subsequently, cells were incubated at 37 °C for two hours with the compounds at indicated concentrations dissolved in vehicle (H_2O, acetone, ethanol or DMSO). Control cultures were incubated with medium alone or with the vehicle. Final vehicle concentration in control or compound exposed cultures did not exceed 0.2%. After two hours plates were sealed and placed for one hour in a waterbath preheated at exactly 42.5°C. After that, cells were allowed to recover at 37°C for indicated time, followed by analysis of Hsp70 expression as described below.

Mice and in vivo WBH treatment

Female BALB/c mice (9-12 weeks) were purchased from Charles River and housed and fed under standard conditions. Experiments were approved by the Animal Experiment Committee of Utrecht University. Whole body hyperthermia (WBH) treatment was done as described previously (33). In brief, seven days before start of WBH treatment, temperature sensitive transponders (IPTT-300; Plexx, Elst, the Netherlands) were implanted subcutaneously in the dorsal thoracic skin. At start of WBH treatment mice were placed in preheated cages and transferred into a preheated incubator. During the experiment temperature of the incubator was adjusted to maintain a core body temperature of approximately 39°C. Mouse core body temperature was measured at indicated time points by wireless transponder readout (WRS-6006/6007; Plexx). Control mice were handled in the same way but at room temperature. After six hours mice were transferred to cages at room temperature. After 18 hours mice were scarified and spleen cells were isolated followed by analysis of Hsp70 as described below.

Preparation of cell lysates and western blot analysis

Cells were pelleted and washed once with PBS. Subsequently, pellets were resuspended in 2x Laemlli sample buffer (150 mM Tris pH 6.8, 4% SDS, 10% glycerol) with 5% protease inhibitor cocktail (Sigma) and sonicated. Protein content was measured with BCA protein assay according to manufacturer's protocol (Pierce, Perbio Science). Before loading on the gel samples were 1:1 diluted in H_2O with 100 mM dithiothreitol. After this the protein content of the diluted sample was adjusted with 1x Laemlli sample buffer to a final concentration of 1.5 mg ml^{-1}. After 5 min boiling equal protein amounts were loaded on 4-16% Criterion SDS-page gels (Biorad). Proteins were transferred to protean nitrocellulose transfer membrane (Schleicher and Schuell). Subsequently, membranes were probed with rabbit anti-Hsp70 (1 µg ml^{-1}) (34) (a kind gift of Dr. Snoeckx, Maastricht), mouse-anti Hsp60 clone LK2 (0.7 µg ml^{-1}) or as a loading control mouse anti-actin clone AC-40 (0.3 µg ml^{-1}) (Sigma). Blots were subsequently incubated with secondary antibodies: rabbit anti-mouse-peroxidase (0.1 µg ml^{-1}) and swine anti-rabbit-peroxidase (0.3 µg ml^{-1}) (Dako; Glostrup, Denmark). All antibodies were diluted in PBS with 0.05% Tween 20 and 1% non fat dry milk (Biorad). Between the different incubation steps, membranes were washed 4 times with PBS 0.05% Tween 20. Visualization was performed with Supersignal West Pico chemiluminescent Substrate (Pierce) for Hsp60 and actin or Supersignal West dura chemiluminescent Substrate (Pierce) for Hsp70 and enhanced chemiluminescence hyperfilm (Amersham, Diegem, Belgium).

Flow cytometric analysis of surface markers and Hsp70 expression

Single cell suspensions of spleen cells were stained with monoclonal antibodies to CD3, CD19 or MHC class II (BD Pharmingen) in PBS plus 2% FBS. For analysis of intracellular Hsp70 expression, cells were fixed and permeabilized for 20 minutes with Cytofix/Cytoperm solution (BD Pharmingen), washed and then incubated with either antibody to Hsp70

fluorescein isothiocyanate labelled (SPA810; Stressgen), that specifically recognizes inducible Hsp70 (HspA1A/HspA1B), or with the corresponding isotype control in Permwash (BD Pharmingen) supplemented with 2% normal mouse serum. For final analysis of fluorescence a FACS-Calibur (BD Pharmingen) was used.

Generation of Hsp70-specific T cell hybridomas

$CD4^+$ T cell hybridomas were generated in our laboratory. In brief, BALB/c mice were immunized on day 0 and day 14 with an immunodominant mouse Hsp70 peptide (mC1b, HspA1A amino acid positions 169 to 183). On day 28 spleen cells were isolated and restimulated with 4 µg ml^{-1} mC1b in X-vivo-15 medium (BioWhitaker) supplemented with glutamax, 100 units ml^{-1} penicillin and 100 µg ml^{-1} streptomycin. After 48 hours, viable cells were isolated using LympholyteM (Cedarlane Laboratories) and cultured for another 48 hours in conditioned medium (IMDM supplemented with 10% FBS, 100 units ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, $5x10^{-5}$ M 2-ME and 10% ConA-activated rat spleen supernatant as a source of T cell growth factors). Subsequently, spleen cells were fused with the fusion partner BW5147 as described previously (35). By FACS Vantage (BD) fused cells were seeded into 96 wells plates at one cell per well. Analysis of mC1b specificity of the obtained clones was performed by incubation of hybridoma cells ($2x10^4$ per well) with APC (A20 B lymphoma cells, $2x10^4$ per well) in 96 wells flat bottom plates for 24 hours with or without peptides at indicated concentrations. Additionally, A20 cells were either or not heat shocked (one hour in a water bath at 42.5°C) followed by 2-3 hours of recovery at 37°C and were afterwards used as APC. Incubation with carvacrol was done for two hours at 0.2 mM prior to heat shock treatment. After overnight, 24 or 48 hours culturing, harvested supernatants were cultured with IL-2 responder CTLL-16 cells for 24 hours, pulsed overnight with [^{3}H]-thymidine (0.4 µCi per well; Amersham Biosciences Europe GmbH), harvested and [^{3}H]-incorporation was measured on a Wallac 1450 MicroBeta liquid scintillation counter. Alternatively, the hybridomas were pulsed overnight with [^{3}H]-thymidine followed by analysis of incorporation. Apoptosis of the hybridomas was measured by incubation with 7AAD- and annexin V-specific antibodies according to manufacturer's protocol (BD). The 5/4E8 hybridoma (36) specific for PG_{70-84}, a proteoglycan-derived peptide, was used as control.

Construction of stable DNAJB1-luc-O23 cell line

A fragment containing the sequence from -500 to +41 of the human DNAJB1 (Hsp40) gene was amplified from genomic DNA using the primers aagtcgaccagacacaggttaggtagttcgtcc and accatggcccctcctgcggcccgccga and cloned SalI/NcoI in the XhoI and NcoI sites of pGL3basic (Promega). The NheI/BamHI fragment of DNAJB1-luc was cotransfected with a TK-hygromycin construct into O23 cells. Stable transfectants were selected and single clones were tested for inducible expression of luciferase. DNAJB1-Luc-O23 cells were kept in culture at 37 °C in DMEM containing 10% FBS, p/s and 1 mg ml^{-1} hygromycin (Roche Diagnostics). For the DNAJB1-luciferase assay, DNAJB1-Luc-O23 cells were seeded at

$5x10^4$ cells per well into 96-well white µClear-plates (Greiner Bio-One) and cultured at 37 °C in DMEM containing 10% FBS and p/s without hygromycin. On the next day, arsenite and carvacrol were added to the indicated concentrations. After 16 hours of overnight incubation, luminescence was measured with the Promega Steady-Glo Luciferase Assay System and counted on a 6-detector Wallac 1450 MicroBeta liquid scintillation counter. Data are expressed as 10^6 counts per minute (10^6 cpm).

Statistical analysis

Statistical analysis was carried out using Prism software (version 4.00, Graphpad Software Inc.). Significance level was set at ($p < 0.05$). Significant differences were calculated by one way ANOVA (two tailed) with Bonferroni correction for multiple comparisons.

Results

Hsp60 and Hsp70 are differentially induced in immune cells by raised temperature

It is important for immune regulation and modulation of inflammation that endogenous Hsp up-regulated during inflammatory stress are recognized. To study if a bona fide stress signal influenced Hsp60 (mitochondrial chaperonin) and/or Hsp70 levels in immune cells, spleen cells from naïve BALB/c mice were exposed to a one hour heat shock (HS) at 42.5℃. After three of hours recovery at 37℃, Hsp60 and Hsp70 protein expression were determined by western blot analysis. In spleen cells incubated at 37℃ Hsp70 expression was very low; a HS treatment resulted in a strong increase (Figure 1A). In contrast, Hsp60 expression was not influenced by HS. Then we questioned if these in vitro findings were relevant for the stress response upon in vivo stress encounter. Therefore, whole body hyperthermia (WBH) of BALB/c mice was induced for six hours at fever range temperature (39℃) as described previously (33), subsequently mice were kept overnight at room temperature.

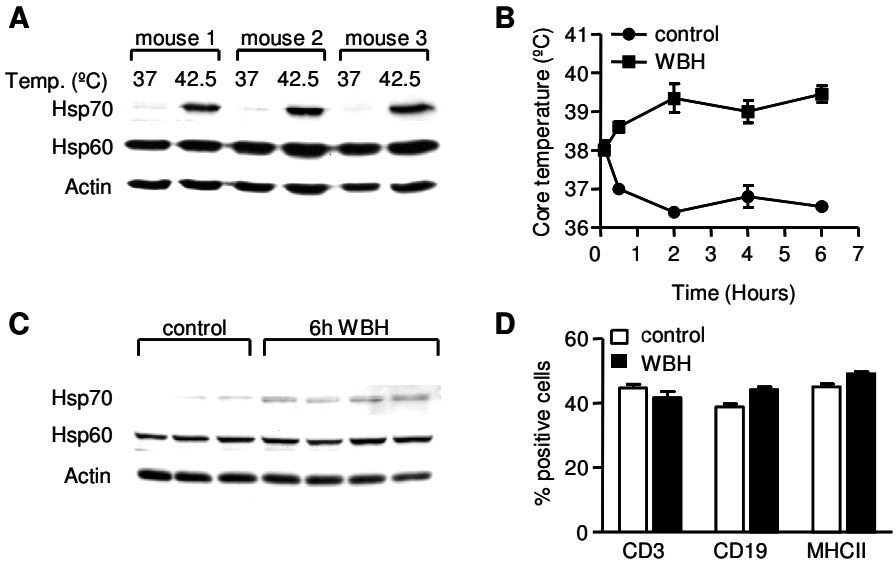

Figure 1. Hsp60 and Hsp70 are differentially induced in immune cells by raised temperature. (A) Primary spleen cells were isolated and heat shocked at 42.5℃, control cells were kept at 37 ºC. After three hours of recovery at 37℃ Hsp60 and Hsp70 expression was measured by western blot analysis. Actin was used as loading control. (B-D) Mice were whole body hyperthermia (WBH) treated for 6 hours in an incubator at fever range temperatures (39℃), control mice were kept at room temperature. (B) Core body temperature was measured by implanted transponders and expressed as mean (± s.d.) of 2 mice. (C) Hsp60 and Hsp70 protein levels measured by western blot analysis of cell lysates obtained from spleen cells of mice after 18 hours of recovery at room temperature. Data show 3 (control) and 4 (WBH) mice. (D) 18 hours after WBH spleen cells were isolated and surface marker expression of CD3, CD19 and MHC class II were determined by flow cytometry. Data are expressed as mean (± s.e.m.) percentage positive cells of total spleen cells obtained from n=3 (control) or n=4 (WBH) mice per group.

WBH enhanced core body temperature of the mice up to 39°C (± 0.5) (Figure 1B). Remarkably, the temperature of control mice also increased immediately after handling of the mice, but rapidly returned to normal. In spite of this short temperature peak, Hsp70 was almost undetectable in spleen cells from control mice. In contrast, the WBH treatment did increase the expression of Hsp70 (Figure 1C). In agreement with our *in vitro* results, no effect on Hsp60 levels was detected. Since *in vitro* heat shock has been reported to induce Hsp with different kinetics and optimal temperatures in T and B cells (37), the difference between control and WHB treated spleen cells could be due to a selective effect of the WHB on the spleen cell population. We thus isolated spleen cells 18 hours after *in vivo* WBH and measured surface expression of CD3, CD19 and MHC class II by flow cytometry. WBH treatment did not significantly influence the percentages of CD3, CD19 or MHC class II positive cells (Figure 1D) illustrating that effects on Hsp expression were not due to different cell populations between control and WBH mice. Therefore, our data show that both *in vitro* and *in vivo* heat treatment increase the expression of Hsp70 but not of Hsp60.

Analysis of Hsp70 expression by flow cytometry

Since we did not detect any effect on Hsp60 expression after heat shock while Hsp70 levels were clearly stress inducible, we focused on Hsp70 expression to analyze the effect of candidate compounds on the heat shock response. Earlier studies that addressed compound induced manipulation of the heat shock response also measured Hsp70 expression (28, 38, 39), enabling easy comparison of previously identified compounds with new ones. First, we addressed whether flow cytometry could be used to study stress induced Hsp70 in several mouse cell types in a simple and rapid manner (40). We analyzed Hsp70 expression in RAW264.7 macrophages, m-IC_{c12} intestinal crypt cells, primary bone marrow derived dendritic cells (BMDC) and spleen cells. After one hour HS at 42.5°C cells were allowed to recover for 18 hours at 37°C, after which Hsp70 expression was analyzed by intracellular staining and flow cytometry. Although the exact response to HS depended on the cell type, increased Hsp70 expression was observed in all cell types as compared to control cultures (Figure 2A). In addition, analysis of the kinetics of Hsp70 expression in these cell types revealed that HS-induced Hsp70 levels peaked between four hours and overnight recovery (Figure 2B-C). Next, we tested whether flow cytometry can be used to detect an effect of Hsp co-inducing compounds on Hsp70 levels by incubation with curcumin or geldanamycin, both compounds known to induce the expression of Hsp70 (21, 39). Curcumin is the active component of the seasoning turmeric, and geldanamycin is an Hsp90 inhibitor produced by Streptomyces hygrocopius. RAW264.7 cells were either incubated for two hours with one of the compounds followed by one hour HS at 42.5°C or only exposed to the HS. After 18 hours of recovery, Hsp70 expression was analyzed. In combination with HS, both compounds enhanced the number of cells expressing Hsp70 as compared to HS treated RAW264.7 cells incubated with medium only or with the vehicle DMSO (Figure 2D-E). In addition, geldanamycin treatment also led to a much higher level of Hsp70 per cell,

while the Hsp70 level per cell after curcumin treatment did not differ much from that in cells just treated with a HS. Without HS both curcumin and geldanamycin slightly augmented the expression of Hsp70 (data not shown). Similar results were obtained in epithelial cells and BMDC (data not shown). In summary, this demonstrated that flow cytometry allowed efficient detection of compound enhanced Hsp70 expression.

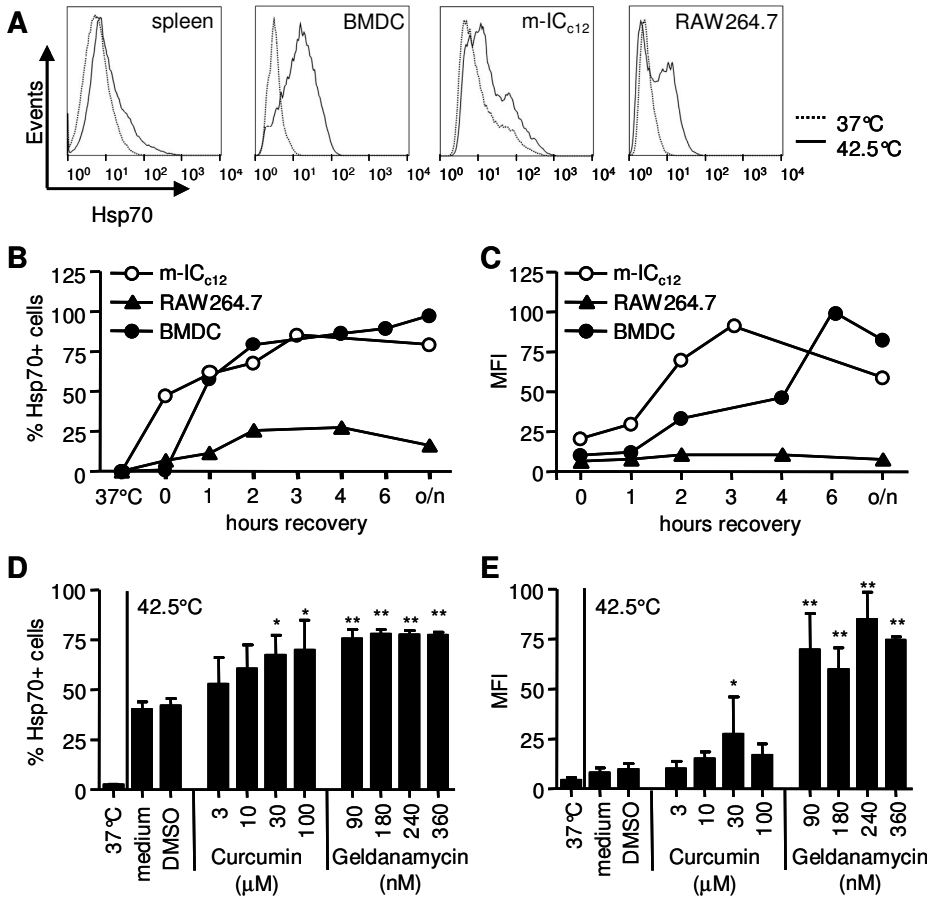

Figure 2. Flow cytometry allows efficient detection of manipulated Hsp70 expression. (A-C) RAW264.7 cells, m-IC$_{c12}$ intestinal cells, primary spleen and bone marrow derived dendritic cells (BMDC) were heat shocked for one hour at 42.5°C or kept at 37°C. After recovery at 37°C for indicated time Hsp70 expression was determined by intracellular staining and flow cytometry. (A) Histograms show Hsp70 expression in viable cells after overnight recovery at 37°C and are representative for at least three independent experiments. (B) % Hsp70$^+$ cells of total viable cells. (C) Mean fluorescence intensity (MFI) of Hsp70$^+$ cells. (D-E) RAW264.7 cells were incubated at 37°C for two hours with curcumin, geldanamycin or the vehicle DMSO (at 0.2%) followed by one hour heat shock at 42.5°C and overnight recovery at 37°C. Graphs show (D) % Hsp70$^+$ cells of total viable cells and (E) mean fluorescence intensity (MFI) of total viable cells. Data are depicted as mean (± s.e.m.) of three independent cultures. * ($p<0.05$) and **(<0.01).

Screening for Hsp-inducing activity of food components

Since we could adequately detect amplified Hsp70 expression after stimulation with Hsp co-inducing compounds by flow cytometry, we used this system to test the effect on Hsp70 expression of eight food extracts that have been described as anti-oxidants (32) and of carvacrol, the major constituent of essential oils obtained from the herbs oregano and thyme (41). For five food extracts both a lipophilic and a hydrophilic fraction was tested. With two extracts, namely black tea and the hydrophilic fraction of saffron, a concentration dependent increase in Hsp70 expression in RAW264.7 cells was seen (Figure 3A-C); the other extracts had no effect on Hsp70 levels (Table 1). None of the extracts had an effect on Hsp70 expression in the absence of an extra stress signal (data not shown). Also incubation with carvacrol did not influence Hsp70 in the absence of stress (data not shown) but carvacrol did amplify Hsp70 expression in combination with stress (Figure 3A-C). Our findings emphasized that our screening system can identify new food-derived compounds acting as Hsp70 co-inducers. Since carvacrol was found superior as co-inducer, in further experiments we focused on carvacrol.

Table 1. Hsp70 co-inducing capacity of food derived extracts

extract		Fold increase (% Hsp70$^+$)	Fold increase (MFI)
Black tea		1.4	4.2
Dark chocolate		2.6	1.2
Milk chocolate		2.3	1.2
Blue berry	**H**	1.2	1.5
	L	1.1	1.4
Lettuce	**H**	1.0	0.4
	L	1.0	1.0
Orange	**H**	1.3	1.2
	L	1.2	1.1
Saffron	**H**	2.3	3.5
	L	0.9	1.0
Spinach	**H**	3.2	1.5
	L	0.8	1.0
H_2O		0.8	0.9
acetone		0.8	1.0

RAW264.7 were incubated with the extracts at 250 μg ml^{-1} (lettuce and orange) 25 μg ml^{-1} (all other extracts) for two hours at 37 °C. Of most extracts two fractions were tested; H indicates the hydrophilic fraction, dissolved in H_2O while L indicates lipophilic fraction dissolved in acetone. As a control cells were incubated with H_2O or acetone alone. In all cultures final H_2O or acetone concentration did not exceed 0.2%. Control cells were incubated with medium. After this incubation cells were cultured for one hour at 42.5°C. Then, cells were allowed to recover overnight at 37°C after which cellular Hsp70 expression was measured by flow cytometry. Data show fold induction as compared to heat shock treatment alone of the percentage of Hsp70$^+$ cells or of mean fluorescence intensity (MFI) of total viable cells.

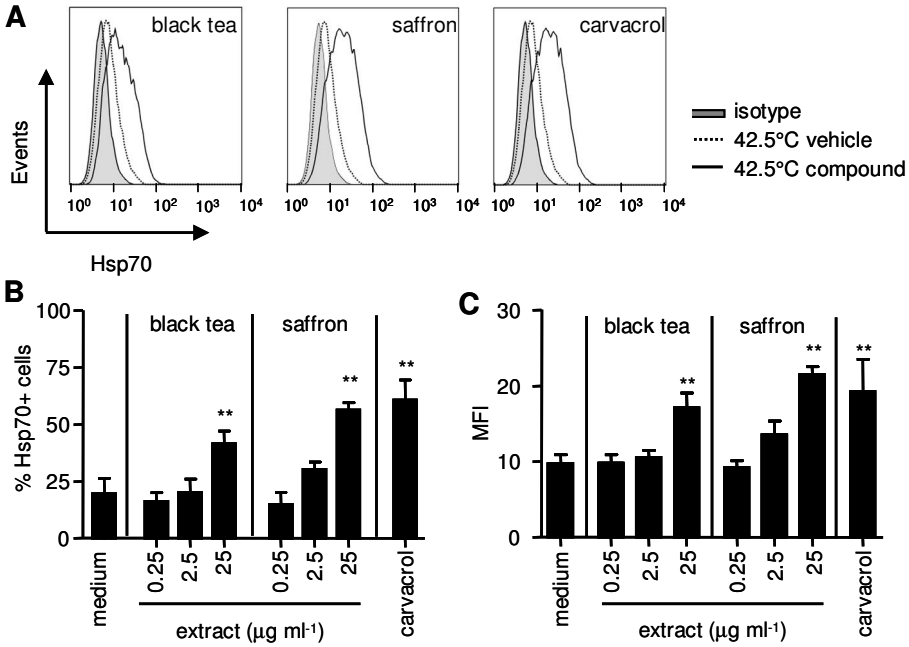

Figure 3. Extracts of black tea and saffron and carvacrol co-induce Hsp70 expression. Incubation of RAW264.7 cells with hydrophilic fractions of black tea and saffron extracts or 0.2 mM carvacrol was done for two hours at 37°C, followed by one hour heat treatment at 42.5°C. After overnight recovery at 37°C intracellular Hsp70 was measured by flow cytometry. (A) Histograms show Hsp70 expression in viable cells. Graphs show (B) % Hsp70$^+$ cells and (C) mean fluorescence intensity (MFI) of total viable cells. Data in B and C show means (± s.e.m.) of triplicate cultures and are representative of two independent experiments. Statistical differences **(<0.01) were calculated by ANOVA (two tailed) with Bonferroni correction for multiple comparisons.

Detection of Hsp70 manipulation by Hsp70-specific CD4$^+$ T cell hybridomas

It is unclear whether increased expression of endogenous Hsp70 also leads to increased presentation of Hsp70-derived peptides on MHC class II. Peptide presentation is essential for activation of Hsp70-specifc CD4$^+$ T cells and thus for Hsp70 dependent immunoregulation. To enable studies on the effect of manipulation of endogenous Hsp70 expression on Hsp70-specific T cell activation, we generated T cell hybridomas from mice that were immunized with a conserved Hsp70 peptide, aa 169-183 from mouse HspA1A (designated mC1b). After limiting dilution cloning of the generated hybridomas 29 clones (designated LHEP1-29) were obtained and tested for mC1b peptide specificity by incubating the clones for 24 hours with the mC1b peptide and irradiated APC (A20). Whether bioactive IL-2 was produced by the hybridoma upon recognition of the peptide was then determined by incubation of harvested hybridoma supernatants with the IL-2 indicator line CTLL-16. Incubation with the peptide enhanced the production of IL-2 in almost all clones as illustrated

in figure 4A for clone LHEP7. We also determined proliferation of the hybridomas themselves by detection of [^{3}H]-thymidine incorporation. Remarkably, augmented production of IL-2 coincided with decreased hybridoma proliferation (Figure 4B). For some T cell hybridomas activation induced cell death (AICD) has been reported (42). We tested this for our hybridomas by 7AAD and annexin V staining and found that incubation with mC1b reduced the percentage of viable, 7AAD$^{neg.}$annexinV$^{neg.}$ cells, while the percentage of non viable 7AAD^{+}annexinV^{+} cells was increased (Figure 4C). Both IL-2 production and AICD could be specifically blocked by and blocking antibody to MHC-II (data not shown).

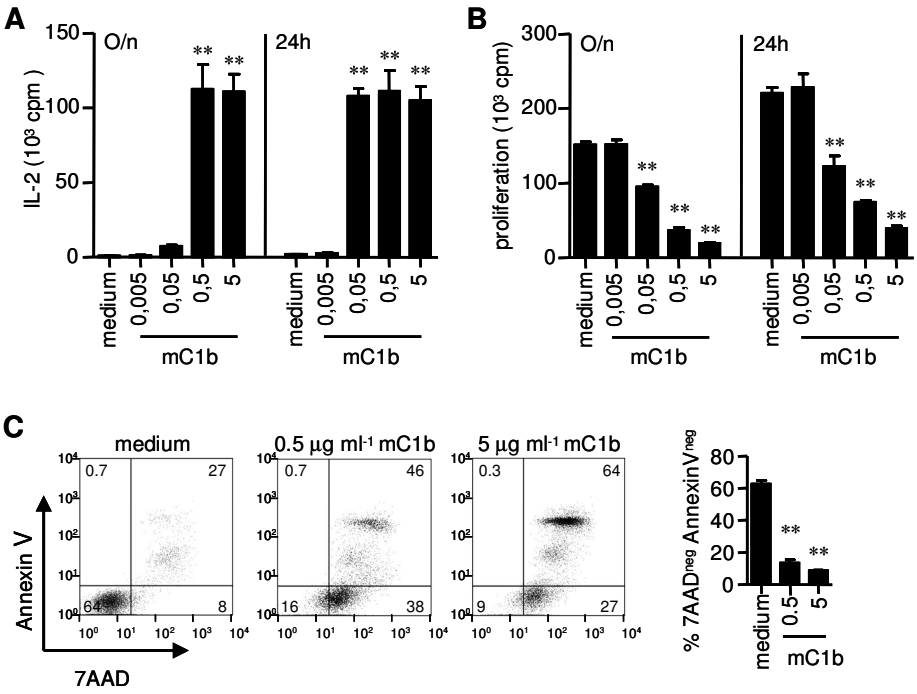

Figure 4. Hsp70-specific CD4^{+} T cell hybridomas show enhanced IL-2 production and reduced proliferation upon activation. Hybridomas specific for the immunodominant Hsp70 mC1b peptide (aa 169-183) were generated by immunization of BALB/c mice with the peptide. Subsequently, fusion of spleen cells isolated from these mice with BW5147 cells was followed by limited dilution cloning of CD4^{+} T cell hybridomas. LHEP7, as representative example of obtained clones, was incubated for 24 hours with antigen presenting cells (A20). To some cultures mC1b peptide at indicated concentrations was added. (A) Bio-active IL-2, produced upon hybridoma activation, was determined as increased [^{3}H]-incorporation by the IL-2 dependent CTLL-16 after overnight culture with harvested hybridoma supernatants. (B) LHEP7 proliferation assayed by incorporation of [^{3}H]-incorporation. Data in A-B are expressed as mean 10^3 cpm (± s.e.m.) of triplicate cultures and are representative of least three experiments. (C) Activation-induced cell death measured by flow cytometric analysis. Graph shows % viable 7AAD$^{neg.}$ and annexin V^{neg} cells of total CD4^{+} cells. Significant differences in A-C as compared to medium incubation are indicated by ** ($p<0.01$).

Then, we studied whether the hybridomas can recognize the endogenous Hsp70. We exposed APC to one hour HS and let them recover for three hours at 37°C. Subsequently, hybridoma clones were stimulated with the HS treated APC. Most clones did not respond to enhanced Hsp70 expression by the APC while others, like LHEP7 and LHEP8, showed enhanced production of IL-2 (Figure 5). As a specificity control we used the 5/4E8 hybridoma (36) that recognizes a proteoglycan peptide (aa 70-84). This hybridoma did not increase production of IL-2 after incubation with HS APC. Incubation of the 5/4E8 hybridoma with the $PG_{(70-84)}$ peptide did increase IL-2 production (data not show). Thus the $CD4^+$ hybridoma T cell clones we isolated differ in recognition of endogenous Hsp, even though they all recognize the mC1b peptide.

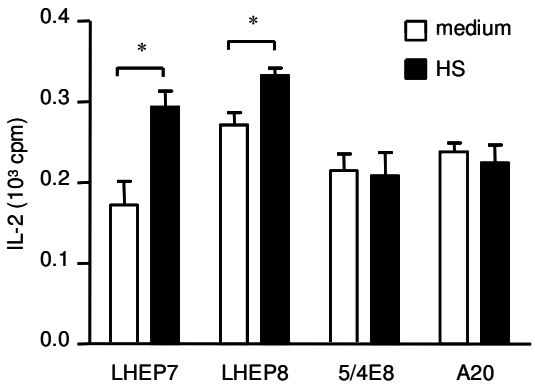

Figure 5. Hsp70-specific $CD4^+$ T cell hybridomas recognize endogenous Hsp70. A20 cells were incubated at 42.5°C for one hour, or kept at 37°C (medium). After three hours of recovery at 37°C, A20 cells were used as APC to stimulate LHEP7 and LHEP8. As a specificity control the 5/4E8 T cell hybridoma recognizing a proteoglycan peptide (aa 70-84) was used. After 24 hours of culture IL-2 production in harvested supernatants was determined by incubation with the IL-2 dependent CTLL-16 cell-line. Data are expressed as mean 10^3 cpm (± s.e.m.) of triplicate cultures and are representative of two independent experiments. * ($p<0.05$).

Hsp70-specific T cell hybridomas respond to compound enhanced Hsp70 expression

In the initial experiments several clones were found to be responsive to enhanced endogenous Hsp70. Therefore, we used some of these hybridomas to test if T cells can respond to manipulation of Hsp70 expression with a co-inducer. APC were incubated with or without 0.2 mM carvacrol for two hours followed by one hour HS at 42.5°C and three hours of recovery at 37°C. Control APC cells were kept at 37°C without carvacrol. Incubation of the hybridomas with HS treated APC increased IL-2 production (Figure 6) to some extent as compared to control APC. Carvacrol pre-treatment of APC augmented the response observed with HS APC. The 5/4E8 hybridoma did not show augmented IL-2 production upon incubation with HS or carvacrol and HS treated APC. The data show that mC1b-specific hybridomas can be used to detect increased presentation of Hsp70-derived peptides in the MHC class II after compound induced up-regulation of the endogenous protein.

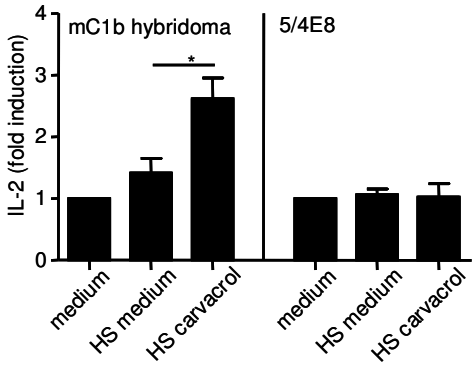

Figure 6. Hsp70-specific T cell hybridomas can respond to compound induced up-regulation of Hsp70. Antigen presenting cells (A20) were incubated or not with 0.2 mM carvacrol for two hours followed by one hour heat shock at 42.5°C. Control cells were kept at 37°C. After three hours of recovery A20 cells were cultured for 24 hours with hybridomas. IL-2 production was determined as described in figure 5. Data are expressed as mean induction (± s.e.m.) relative to medium non-HS treatment (cpm medium/cpm HS or HS + carvacrol). mC1b data show pooled data of LHEP7, LHEP13, LHEP19 and LHEP24. * ($p<0.05$).

The effect of carvacrol on activation of the DNAJB1 promoter

The studies reported above show that carvacrol is a co-inducer of Hsp70 in that carvacrol only augments Hsp70 expression upon stress, not in the absence of stress. To test whether carvacrol sensitizes cells to stress or enhances Hsp70 expression in stressed cells, we turned to arsenite as a stressor and O23 cells carrying a luciferase reporter gene driven by the DNAJB1 (Hsp40) promoter. Arsenite was chosen because it is experimentally easier to control the level of stress by adjusting the concentration of arsenite than by changing the severity of a heat shock; a luciferase reporter system was selected because it is more quantitative than the experimental systems used above; the DNAJB1 promoter was selected as we found that the DNAJB1 mRNA level is more sensitive to inhibition of HSF1 than the level of Hsp70 mRNA (manuscript in prep.). In line with the Hsp70 data obtained by flow cytometry we found that carvacrol dose dependently enhanced the luciferase activity induced by arsenite exposure (Figure 7). For carvacrol to act at least some stress is needed: no effect is seen at 0 and 2.5 μM arsenite while at 5 μM arsenite the luciferase signal is doubled with the highest concentrations of carvacrol tested. These data suggest that carvacrol does not sensitize the cells to stress but enhances the response to stress.

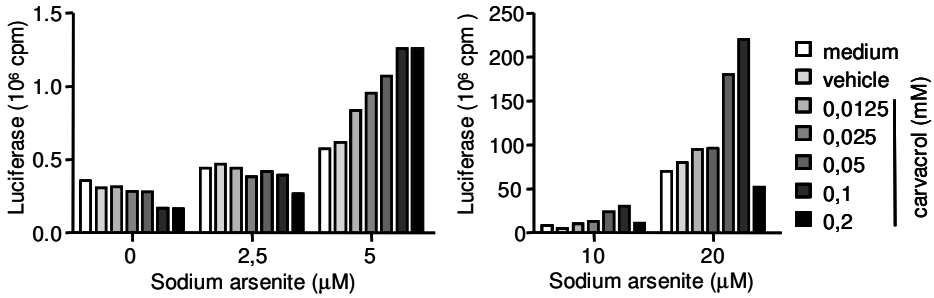

Figure 7. Analysis of HSF1 activation with DNAJB1-luc-O23 reporter cells. Reporter cell lines stably expressing a fragment containing the sequence from -500 to +41 of the human DNAJB1 (Hsp40) gene were generated. Cells were incubated with carvacrol and sodium arsenite at the indicated concentrations at 37°C After overnight incubation, luciferase expression was determined and depicted as 10^6 cpm. Similar data were obtained in >10 experiments.

Discussion

Enhanced expression induced by stress is one of the hallmarks of inducible Hsp family members. This seems important for the protective effects of Hsp, at the level of both the cell itself and the immune system (1, 7, 43). Boosting the heat shock response with food-derived compounds may be an attractive way to modulate Hsp expression. Here we describe three ways to monitor the effect of (co-) inducers of the heat shock response. First, we show that flow cytometry-based analysis of cellular Hsp70 levels is an efficient and reliable way to study the effect of manipulation of the heat shock response. Second, we describe a panel of monoclonal $CD4^+$ T cell hybridomas. These T cell hybridomas specifically recognize an immunodominant mouse Hsp70 peptide in a MHC class II dependent fashion and respond to enhanced Hsp70 levels in APC. Third we generated a luciferase reporter system to investigate if compounds sensitize cells to stress or enhance the stress response.

We first set out to study if Hsp were up-regulated in rodent immune cells both *in vitro* and *in vivo* in response to a regular stress signal. Hsp70 but not Hsp60 expression was increased upon *in vitro* HS treatment in spleen cells isolated from BALB/c mice. This was confirmed after *in vivo* heat shock treatment for six hours at fever range temperatures. In a previous study enhanced Hsp70 and Hsp110 have been found upon 6 hours WBH (44). Furthermore, using a more severe WBH protocol, 20 min at 41°C, in rats enhanced expression of Hsp70 but not Hsp60 has been observed but, Hsp60 was induced upon water immersion stress (45). In contrast, in another study in mice water immersion stress (30 min 41.5°C) did not enhance Hsp60 levels while Hsp70 was increased (46) This underlined that the outcome on Hsp60 expression will depend on the exact stress signal. Hsp70 induction seems less trigger dependent, and shows strong induction by all kinds of stress signals.

In many previous studies analysis of Hsp70 expression has been performed by western blot (28, 38, 39, 47). Firstly, this is a relatively time-consuming technique. Additionally, this technique does not allow analysis of individual cell populations such as B and T cells. Such a distinction can be made with flow cytometry, where Hsp70 staining can be combined with surface marker staining of specific cell populations. We explored the potential of flow cytometry to detect Hsp70 expression and found that, although induced Hsp70 levels differed between the various cell types, elevated Hsp70 levels were found in all cell types after HS treatment as compared to control cultures. Variation between cell types in Hsp70 expression and kinetics thereof have been described before (37). Therefore, we also tested milder HS protocols (0.5 hours at 42.5°C or one hour at 39°C) and observed slightly enhanced Hsp70 levels compared to control cultures. But the day to day variation was higher then observed with the one hour HS at 42.5°C protocol. In agreement with previous literature Hsp70 expression was increased after pre-incubation of RAW264.7 cells with geldanamycin (21) or curcumin (39) in combination with HS. This proved that compound

induced Hsp70 expression can be successfully detected by flow cytometry. Additionally, we discovered the Hsp70 co-inducing capacity of extracts of saffron or black tea and carvacrol, demonstrating that we can also identify new Hsp co-inducers.

An important aspect of immunoregulation is the activation and/or induction of antigen-specific regulatory T cells. Different regulatory T cells require different means of activation. To activate Hsp-specific $CD4^+$ T cells, augmented expression of the endogenous Hsp70 should lead to increased presentation of Hsp70 peptides on MHC class II. Also, Hsp can act as so called ergotopes; antigenic determinants derived from activation markers including CD25 and Hsp. Up-regulation of these ergotopes on activated T cells can activate anti-ergotypic T cells with regulatory capacity. Most anti-ergotypic T cells subsets recognize ergotopes in an MHC restricted manner but also MHC independent induction of gamma-delta T cells with regulatory capacity has been described (48, 49). In the present study however we focused on $CD4^+$ T cells. To study this, we generated $CD4^+$ T cell hybridomas that recognized an Hsp70-derived peptide (mC1b). Recently, we found mC1b to suppress inflammation in a rodent arthritis model (manuscript in preparation). In the present study, multiple mC1b-specific $CD4^+$ T cell hybridomas were obtained. While addressing hybridoma recognition of the endogenous Hsp70, we found that some of the hybridoma clones were increasingly activated after incubation with HS APC. In line with this data, our previous study in rats showed that Hsp60-specific T cells recognized upregulation of cellular Hsp60 in the APC (50). The mC1b peptide from mouse HspA1A has been frequently eluted from MHC class II (51-53). Our findings presented here show that these peptides were visible to Hsp70-specific T cells. Activation of the hybridomas was increased when carvacrol treated APC were used, as compared to control or HS only APC. Therefore, our results also illustrated that Hsp70-specific hybridomas can be used as read out for Hsp70-derived peptide presentation upon manipulation of the stress response.

Besides Hsp70, some DNAJ family members are stress inducible and also regulated by HSF1 (5, 54, 55). In the present study we tested a DNAJB1 reporter cell line that allowed very fast analysis of DNAJB1 promoter activity. Using carvacrol, we found that this line can be used to screen for the effects of candidate compounds on DNAJB1 expression. Previously, Hsp60- or Hsp60 peptide-specific T cell responses have been associated with disease remission and a favourable disease outcome in patients with juvenile idiopathic arthritis or rheumatoid arthritis (56, 57). Recently, the immunomodulatory effects of T cell epitopes from DNAJ proteins of both human and *Escherichia coli* origin have been studied in juvenile idiopathic patients (58). In that study, T cell responses to bacterial peptides were shown to be pro-inflammatory. In contrast, responses to human peptides had a more regulatory phenotype and were associated with a better disease prognosis. Furthermore, DNAJ-like2 was one of the broadly expressed self-antigens that resulted in the development of $CD4^+CD25^+$ regulatory T cells after immunization of BALB/c mice (59). Also enhanced expression of DNAJ family members in the synovial tissue of RA but OA patients has been

found (60). These studies underlined that compounds acting on DNAJ and Hsp70 expression might also target both DNAJ- and Hsp70-specific T cell immunity, both with inflammation modulatory capacity.

In aged individuals the stress induced expression of Hsp has been reported to be decreased (61, 62). Because expression of Hsp at the inflammatory site is probably important for the regulatory function of Hsp-specific T cells this reduced expression might add to the increased susceptibility of aged individuals for inflammatory disease. Boosting or restoration of a failing heat shock response could restore immune regulation. Food-derived compounds will enable relatively safe and non-toxic interference with the stress response. The systems described in the present study, will enable further studies on the effects of new and already identified enhancers of the heat shock response.

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CHAPTER 4

IL-10 is critically involved in mycobacterial Hsp70-induced suppression of proteoglycan-induced arthritis

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Abstract

The anti-inflammatory capacity of heat shock proteins (Hsp) has been demonstrated in various animal models of inflammatory diseases and in patients. However, the mechanisms underlying this anti-inflammatory capacity are poorly understood. Therefore, the possible protective potential of Hsp70 and its mechanisms were studied in proteoglycan (PG) induced arthritis (PGIA), a chronic and relapsing, T cell mediated murine model of arthritis.
Hsp70 immunization, 10 days prior to disease induction with PG, inhibited arthritis both clinically and histologically. In addition, it significantly reduced PG-specific IgG2a but not IgG1 antibody production. Furthermore, IFN-γ and IL-10 production upon *in vitro* restimulation with Hsp70 was indicative of the induction of an Hsp70-specific T cell response in Hsp70 immunized mice. Remarkably, Hsp70 treatment also modulated the PG-specific T cell response, as shown by the increased production of IL-10 and IFN-γ upon *in vitro* PG restimulation. Moreover, it increased IL-10 mRNA expression in $CD4^+CD25^+$ cells. Hsp70 vaccination did not suppress arthritis in $IL\text{-}10^{-/-}$ mice, indicating the crucial role of IL-10 in the protective effect.
In conclusion, a single mycobacterial Hsp70 immunization can suppress inflammation and tissue damage in PGIA and results in an enhanced regulatory response as shown by the antigen-specific IL-10 production. Moreover, Hsp70 induced protection is critically IL-10 dependent.

Introduction

Heat shock proteins (Hsp) are highly immunogenic and have the potential to trigger immunoregulatory pathways. Although the exact mechanisms of immunoregulation by Hsp remain to be clarified, T cells specific for Hsp were suggested to be involved in regulation of multiple chronic inflammatory diseases like rheumatoid arthritis (RA), diabetes and atherosclerosis (1-4).
Recent studies on Hsp-responding T cells of juvenile idiopathic patients implied a regulatory role and contribution to disease remission for Hsp60-specific T cells (5, 6). Among the various Hsp families of molecules, the immunomodulatory potential of Hsp60 has been studied most extensively. However, also other Hsp have immunoregulatory functions. For example treatment with bacterial-Hsp70 has been shown to protect against development of adjuvant-induced arthritis in rats, whereas treatment with other highly conserved bacterial proteins did not (7). Exposure to bacterial Hsp has been shown to activate self-Hsp-specific T cells that were cross reactive with bacterial-Hsp and induced suppression of arthritis (8, 9). In addition, a T cell line specific for an Hsp70-derived peptide has been described to decrease disease severity upon transfer (10). The protective potential of Hsp, in animal models, has been reproduced by recent clinical trials with Hsp-derived peptides in patients (11, 12).
Even though Hsp-induced immunoregulation has been studied rather extensively, the mechanism of Hsp-induced protection in autoimmune diseases is still not clear. A potential regulatory mechanism might be via the induction of IL-10 since the important role of IL-10 in dampening inflammation has been described extensively (13-15). Additionally, IL-10 requirement for their suppressive function has been thought to be a common feature among most subsets of regulatory T cells as summarized (16).

In the present study the protective potential of mycobacterial-Hsp70 immunization on inflammatory disease and its dependency on IL-10 were assessed in the proteoglycan-induced arthritis model (PGIA), a progressive T cell dependent, antibody-mediated murine model for RA (17). In this model arthritis can be induced by immunization with human proteoglycan (PG) mixed with synthetic adjuvant dimethyldioctadecylammonium bromide (DDA) instead of complete Freund's adjuvant (CFA) (18). Therefore, the possibility of interfering with immune responses induced by mycobacterial-Hsp present in CFA can be excluded. We found that Hsp70 immunization dramatically suppressed arthritis development, subsequent tissue damage and the pathogenic PG-specific antibody response. Moreover, we demonstrate that Hsp70 immunization results in a regulatory T cell response not only to Hsp70, but also to the disease inducing PG. Finally, Hsp70 did not suppress arthritis in IL-10 deficient mice, indicating that the regulatory response is IL-10 dependent.

Materials and Methods

Mice

Female BALB/c mice, retired breeders aged between 16-26 weeks, purchased from Charles River (Maastricht, The Netherlands), and IL-$10^{-/-}$ mice also on the BALB/c background (kindly provided by Dr. D. Reddick, DNAX, Palo Alto, CA) (19) were bred, housed and fed under standard conditions. Experiments were approved by the Animal Experiment Committee of Utrecht University (Utrecht, the Netherlands).

Induction and assessment of arthritis

Arthritis was induced with PG using a standard immunization protocol as described (17, 18). Briefly, 300 µg PG protein was given by intra peritoneal injection (i.p) with 2 mg of DDA (Sigma) emulsified in 200 µl PBS on days 0 and 21. PG was prepared as described elsewhere (20). After the second PG immunization the paws of mice were examined in a blinded fashion 3 times a week to record arthritic changes of the joints. The onset and severity of arthritis were determined using a visual scoring system based on swelling and redness of paws as described (18). Upon sacrifice, joints were fixed in 10% buffered formalin, decalcified in 0.33 M neutralized EDTA, embedded in paraffin and 5 µm sagittal sections were stained with hematoxylin and eosin.

Hsp70 or control immunization

Hsp70 or control immunization was done, 10 days prior to arthritis induction, by intraperitoneal (i.p.) immunization with 100 µg recombinant Hsp70 of *Mycobacterium tuberculosis* (Mt) (LIONEX Diagnostics & Therapeutics GmbH), or 100 µg control protein, either recombinant enhanced green fluorescent protein (EGFP) or ovalbumin (Ova) (Sigma) in adjuvant DDA, 2 mg emulsified in PBS or with PBS in a total volume of 200 µl. To avoid interference of LPS contamination with Hsp70 treatment, Hsp70 containing less than 2.1 EU/mg protein was used.

Analysis of PG- and Hsp70-specific serum antibody production

Antigen-specific serum antibody levels were determined by ELISA as described previously (18). In brief, 96 well plates (Corning) were coated by overnight incubation with 100 µl PG or Hsp70 at 5 µg/ml in coating buffer (0.1 M $NaHCO_3$, Na_2CO_3 pH 9.6). Free binding sites were blocked with blocking buffer, Roche blocking reagents for ELISA (Roche Diagnostics) followed by incubation with sera at increasing dilutions and subsequently peroxidase-conjugated anti-IgG1, -IgG2a or -total IgG (BD Pharmingen) in blocking buffer. After ABTS incubation serum PG-specific antibody levels were calculated as OD relative to the OD measured for the corresponding isotypes of a standard of pooled sera from arthritic mice. For Hsp70-specific antibody production a reference serum of Hsp70 immunized mice was used. Data were expressed relative to the average of the control group.

Analysis of antigen-specific T cell responses

Single-cell suspensions of spleens were cultured in triplicates in 96-well flat bottom plates (Corning) at $2x10^5$ cells per well, in the presence or absence of Hsp70 (10 μg/ml), PG (10 μg/ml) or Ova (10 μg/ml). Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% FBS (Bodinco), 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and $5x10^{-5}$ M 2-mercaptoethanol was used as culture medium. After 72 or 96 hours, the cells were pulsed overnight with ^{3}H-thymidine (0.4 μCi per well; Amersham Biosciences Europe GmbH), harvested and ^{3}H uptake was measured by liquid scintillation counting (Microbeta, Perkin-Elmer Inc.). Supernatants of antigen stimulated spleen cell cultures were collected for cytokine assays after 72 hours. Supernatants harvested from cultures with cells isolated from WT mice were analyzed for IL-10 and interferon-gamma (IFN-γ) expression simultaneously by multiplex analysis using the Luminex 100 system (Becton Dickinson, Mountain View, CA). The LINCOplex assay was performed according to the manufacturer's instructions (Linco Research, Inc.). The concentrations of IL-10 and IFN-γ in the supernatants were calculated using LMAT software (Luminex Corporation). IFN-γ secretion by cells isolated from $IL10^{-/-}$ mice was measured by ELISA (BD OptEIA) according to manufactures protocol (BD Pharmingen).

Assessment of IL-10 and Foxp3 mRNA expression, in $CD4^+CD25^+$ spleen cells

Isolation of $CD4^+CD25^+$ spleen cells from arthritic mice was done by staining single cell suspensions of spleen cells with anti-CD4-allophycocyanin (RM4-5) and anti-CD25 r-phycoerythrin (PC61) monoclonal antibodies (BD Pharmingen). $CD4^+CD25^+$ cells were sorted by CD4 and CD25 expression with a FACSVantage SE (BD. Then total mRNA extraction, with the RNeasy kit (Qiagen), on column DNAse treatment (Qiagen), and transcription into cDNA using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratries) were carried out according to manufacturers protocol. PCR (3 min at 95 ºC and 40 cycles of 10 s 95ºC and 45 s at 59.5ºC) and Real-Time detection were performed in a Bio-Rad MyiQ iCycler (Bio-Rad). Amplification was done using IQ™ SYBR Green® Supermix (Bio-Rad) with 0.25 μM final concentrations of primers specific for IL-10 (5'-GGT TGC CAA GCC TTA TCG GA-3'and 5'-ACC TGC TCC ACT GCC TTG CT-3'), Foxp3 (5'- CCC AGG AAA GAC AGC AAC CTT and 5'-TTC TCA CAA CCA GGC CAC TTG-3) and hypoxanthine-guanine phosphoribosyl transferase (HPRT) (5'-CTG GTG AAA AGG ACC TCT CG and 5'-TGA AGT ACT CAT TAT AGT CAA GGG CA-3'). For each sample mRNA expression was normalized to the detected Ct value of HPRT and expressed relative to the average of the control group.

Statistical analysis

Unless stated otherwise, data are expressed as mean ± standard error of the mean (s.e.m.) statistical analysis was carried out using the Mann-Whitney U test (two-tailed) using Prism software (version 3.00, Graphpad Software Inc.). Significance level was set at ($p < 0.05$).

Results

Immunization with Hsp70 decreases arthritis incidence and severity

To investigate the immunoregulatory mechanisms of Hsp70 in inflammatory diseases, the effect of mycobacterial-Hsp70 immunization in PGIA was studied. Mice were treated with Hsp70 10 days prior to induction of arthritis by i.p. immunization with 100 µg Hsp70, in the synthetic adjuvant DDA, whereas control groups received 100 µg EGFP or PBS in DDA. Subsequently, arthritis was induced by two i.p. immunizations with PG in DDA on day 0 and 21. Hsp70 immunization lowered the incidence of arthritis and significantly delayed the onset of arthritis; day 52 ± 3.2 in Hsp70 immunized mice compared to day 31 ± 2.0 in EGFP and 30 ± 1.0 in PBS pretreated mice ($p<0.01$) (Figure 1A). Furthermore, Hsp70 pre-treatment evidently reduced severity of PGIA (Figure 1B). In association, histological analysis of joint sections of Hsp70 immunized arthritic mice showed very mild leukocyte infiltration, less reactive synovial cell proliferation, and consequently almost no cartilage damage compared to joint sections of control animals (Figure 2A-B).

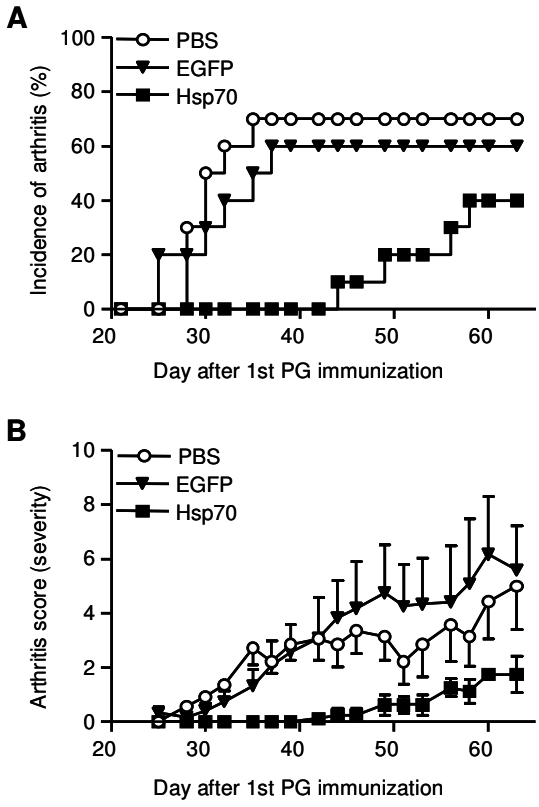

Figure 1. Decreased arthritis incidence and severity after Hsp70 immunization. Mice were injected intraperitoneally, 10 days prior to disease induction, with either 100 µg recombinant mycobacterial heat shock protein 70 (Hsp70) or 100 µg enhanced green fluorescent protein (EGFP) both emulsified in synthetic adjuvant DDA or with PBS. Arthritis was induced by two immunizations of proteoglycan (PG) in DDA on day 0 and 21. (A) Arthritis incidence expressed as the cumulative percentage of arthritic animals and (B) arthritis severity expressed as the mean arthritis score of sick mice ± s.e.m.. Data are representative of three independent experiments (n=10 in each treatment group).

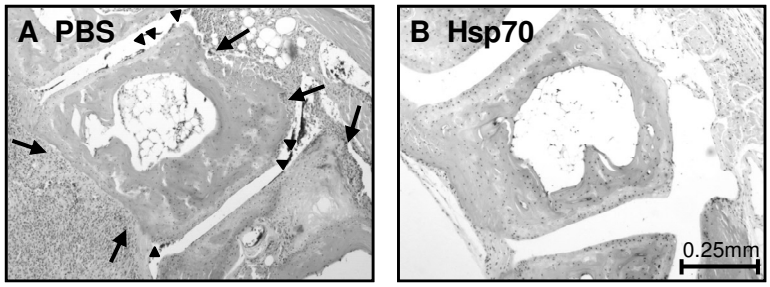

Figure 2. Decreased leukocyte infiltration and cartilage breakdown in Hsp70 immunized mice. PBS or Hsp70/DDA immunization, followed by arthritis induction, was performed as described in figure 1. Upon sacrifice on day 35, histology of tarso-metatarsal joints was done by staining of the sections with hematoxylin and eosin. (A) Synovial hyperplasia with infiltrating leukocytes, cartilage- (arrow heads) and bone-erosions (arrows) can be seen. (B) In contrast, only a very few leukocytes, and no synovial cell proliferation or cartilage destruction were observed in the joint sections of Hsp70 immunized mice.

Altered antigen-specific B cell responses after Hsp70 immunization

PG-specific B cell responses have been described to be required for development of severe PGIA (17). Therefore, the PG-specific B cell response and the effect of Hsp70 immunization on this response were studied. On day 38 after the first PG injection PG-specific serum IgG1 and IgG2a levels were determined by ELISA. High IgG1 levels confirmed the induction of a B cell response against the disease inducing PG in both treatment groups. However, the level of IgG2a was lowered in Hsp70 compared to PBS immunized mice (Figure 3A). In addition, we studied the pattern on days 50-64, after development of mild disease in Hsp70 immunized mice. At days 50-64 the difference between PBS and Hsp70 immunized mice in IgG1 levels was comparable to day 38 but the decrease in IgG2a, as observed on day 38, was no longer significant (Figure 3B). The data indicate that mainly the PG-specific IgG2a production was influenced by Hsp70 immunization and underline the importance of this isotype for induction of PGIA.

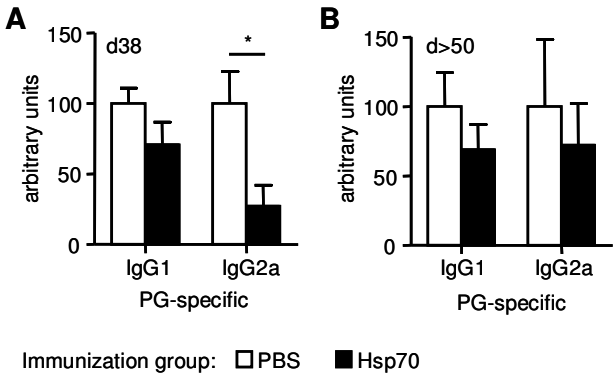

Figure 3. Decreased PG-specific B cell responses in Hsp70 immunized mice. PG-specific IgG1 and IgG2a were determined by ELISA in sera obtained (A) on day 38 or (B) on day 50 or 64 after disease induction. Mean relative serum antibody levels (± s.e.m) were calculated as OD relative to the OD measured for a standard of pooled sera. Data in A (n=5 per group) are representative of three independent experiments. Values in B show means (n=6 mice) of sera obtained on either day 50 or day 64, (* $p<0.05$)

Recently, Hsp-specific antibodies have been shown to play a role in Hsp-induced regulation in models for RA (21, 22). To address whether Hsp70-specific antibodies were also induced upon Hsp70 immunization in the PGIA model, Hsp70 serum antibody levels were analyzed by ELISA. Significantly increased Hsp70-specific total IgG production was found in Hsp70 immunized mice (Figure 4). Thus, both PG- and Hsp70-specific B cell responses were altered upon Hsp70 treatment as shown by a decreased pathogenic PG-specific response and an increased, possibly protective Hsp70-specific B cell response.

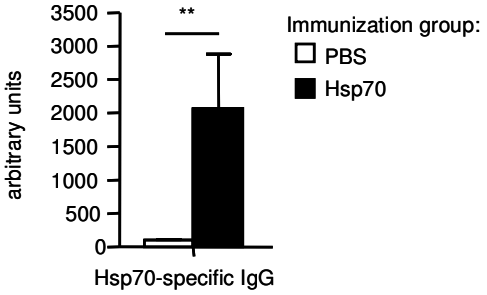

Figure 4. Altered PG- and Hsp70-specific B cell responses in Hsp70 immunized mice. Hsp70-specific total IgG production was determined by ELISA in sera obtained on day 38. Values represent means ± s.e.m. (n=5 mice in each group). Significant difference (**p<0.01, Mann-Whitney U Test) is indicated by the asterisk.

Increased antigen-specific T cell responses after immunization with Hsp70

In previous studies, Hsp-specific T cell responses have been described to be involved in Hsp-induced protection from disease development (7, 9). To study the effect of Hsp70 immunization on differentiation of antigen-specific T cell responses, PG- and Hsp70-specific proliferation and cytokine profiles were analyzed. Therefore, spleen cells from Hsp70 or PBS immunized mice were harvested on day 35 after the first PG immunization. Antigen-specific proliferation was assessed by culturing the isolated cells for 96 hours with PG or Hsp70 while Ova was used as control antigen. During the last 18 hours ^{3}H-thymidine was added and incorporation was measured and expressed as mean stimulation index (cpm antigen/cpm background) ± s.e.m.. The mean background values (responses without antigen) were for the PBS control group 8390 ± 2135 and for the Hsp70 group 10180 ± 2741. A significantly enhanced proliferative response to Hsp70 restimulation was seen in Hsp70 immunized mice as compared to control mice (Figure 5A). Remarkably, also the proliferative response against the arthritis inducing PG was enhanced in the Hsp70 group. Next, antigen-induced production of IL-10 and IFN-γ was studied in supernatants collected after 72 hours culturing in the presence of PG or Hsp70. Increased IL-10 production, by spleen cells from Hsp70 immunized mice, could be detected after Hsp70 *in vitro* restimulation (Figure 5B). Interestingly, this increased IL-10 production was also found after PG *in vitro* restimulation. Furthermore, the amount of IFN-γ produced by Hsp70- and PG-specific cells from Hsp70 treated mice was increased (Figure 5C). In summary this clearly showed that Hsp70 immunization not only induced an Hsp70-specific T cell response but also modulated the T cell response to the disease-inducing antigen.

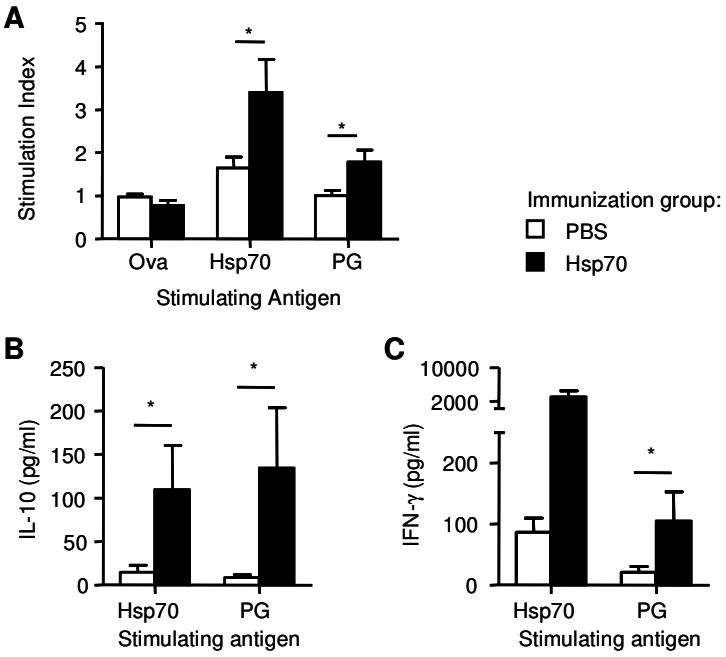

Figure 5. Augmented antigen-specific proliferation, IL-10 and IFN-γ production in Hsp70 immunized mice.
Mice were immunized with PBS or Hsp70 and arthritis was induced as described in figure 1. Spleen cells were isolated on day 35 and cultured with mycobacterial Hsp70, PG or Ova. (A) After 96 hours incubation proliferation was determined by measuring ^{3}H incorporation. Results are expressed as mean stimulation index (cpm antigen/cpm background) ± s.e.m.. The mean background values (responses without antigen) were for the PBS control group 8390 ± 2135 and for the Hsp70 group 10180 ± 2741. (B-C) For *in vitro* cytokine secretion the spleen cells were cultured in the presence of antigen. After 72 hours supernatants were harvested and assayed by multiplex analysis for IL-10 and IFN-γ excretion. Values represent the means ± s.e.m. of cytokine levels. Significant difference ($p<0.05$, Mann-Whitney U Test) is indicated by the asterisk. Data are representative for at least two independent experiments.

Enhanced IL-10 mRNA expression in $CD4^{+}CD25^{+}$ T cells after Hsp70 immunization

It has been proposed that induction or activation of Hsp70-specifc regulatory T cells cross-reactive with self-Hsp70 are involved in protection seen after Hsp70 immunization (8, 9). To study in more detail whether Hsp70 immunization influenced natural regulatory T cells, $CD4^{+}CD25^{+}$ cells were isolated from the spleen on day 38 after the first PG injection. In this population Foxp3 and IL-10 mRNA expression were analyzed by Quantitative Real-Time PCR analysis showing that IL-10 mRNA expression was increased in the $CD4^{+}CD25^{+}$ population from Hsp70 treated mice compared to PBS treated control mice (Figure 6). No clear difference in Foxp3 mRNA expression was observed, between control and Hsp70 immunized mice. This could be confirmed by intracellular staining and flow cytometric analysis of Foxp3 protein expression (data not shown). Indicating that Hsp70 does not increase the population of Foxp3 expressing regulatory T cells.

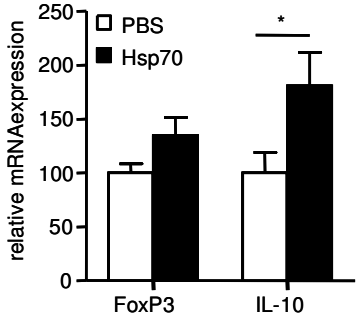

Figure 6. Increased IL-10 mRNA expression in CD4$^+$CD25$^+$ cells of Hsp70 immunized mice. Mice were PBS or Hsp70 immunized before induction of arthritis as described in figure 1. On day 38, CD4$^+$CD25$^+$ cells were isolated from the spleen followed by isolation of mRNA. Foxp3 and IL-10 mRNA expression was determined by Quantitative Real-Time PCR analysis. Data are normalized and expressed relative to HPRT and depicted as means ± s.e.m. (n=5 mice in each group). Significant difference * ($p<0.05$, Mann-Whitney U Test) is indicated by the asterisk.

IL-10 is important for the protective effects of Hsp70 immunization

Hsp70 immunization increased the antigen-specific IL-10 production suggesting that IL-10 is important for the anti-inflammatory capacity of Hsp70. To address whether protection in arthritis by Hsp70 immunization was indeed IL-10 dependent, IL10$^{-/-}$ mice received Hsp70 or Ova as control i.p. in DDA on day -10, followed by induction of arthritis as described in figure 1. Hsp70 immunization of IL10$^{-/-}$ mice, in contrast to wild type mice (WT), did not reduce arthritis severity as compared to control, immunized IL10$^{-/-}$ mice. In addition, there was no obvious difference in the mean maximum arthritis score or the day of onset of disease (Table 1). To study the effect of Hsp70 immunization, in the absence of IL-10, on the B cell response, PG- and Hsp70-specific antibody production was measured in the sera of IL10$^{-/-}$ mice on day 53 after the first PG injection. Induction of arthritis resulted in a clear PG-specific antibody response as shown by elevated IgG1 and IgG2a levels in both treatment groups (Figure 7A). However, in parallel with the arthritis scores, Hsp70 immunization in IL-10$^{-/-}$ mice did not influence this PG-specific response. As expected, Hsp70 immunization enhanced the Hsp70-specific IgG production (Figure 7B). Next, induction and modulation of the antigen-specific T cell response by Hsp70 treatment in IL-10$^{-/-}$ mice were addressed. Therefore, spleen cells were harvested on day 53 followed by analysis of antigen-specific proliferation after *in vitro* restimulation, for 72 hours with PG, Ova or Hsp70. Similar to the response in WT mice, immunization of IL10$^{-/-}$ mice with Hsp70 enhanced the proliferative response to Hsp70 restimulation compared to control immunization with Ova. But, it did not have an effect on PG-specific proliferation (Figure 7C). Furthermore, Ova specific proliferation was slightly increased in mice that received Ova. In addition, the effect of Hsp70 immunization on antigen-specific IFN-γ secretion was studied, by ELISA in the culture supernatants. Also in IL10$^{-/-}$ mice, immunization with Hsp70 increased the production of IFN-γ after Hsp70 restimulation. But, the enhanced PG-induced IFN-γ production, observed in WT mice, was not observed in IL10$^{-/-}$ mice (Figure 7D). Taken together, this indicated that Hsp70 immunization of IL-10$^{-/-}$ mice induced an Hsp70-specific B and T cell response that, in the absence of IL-10, failed to suppress PGIA development and PG-specific pathogenic IgG2a production.

Table 1: Mean day of onset and maximum arthritis score in WT and IL10$^{-/-}$ mice.

	WT			IL10$^{-/-}$	
	Mean day of onset	Maximum arthritis score		Mean day of onset	Maximum arthritis score
control	31 ± 2.0	4.6 ± 1.6		33.6 ± 1.3	5.4 ± 1.1
Hsp70	52 ± 3.2 **	1.1 ± 0.3 *		33.0 ± 0.0	4.2 ± 1.4

Arthritis was induced in WT BALB/c (n=10 per group) or IL-10$^{-/-}$ mice (n=5 per group) by immunizations with PG in the adjuvant DDA on days 0 and day 21. Ten days prior to the induction of arthritis mice were immunized with Hsp70 whereas control mice received EGFP or Ova. Development of arthritis symptoms was scored by clinical examination. Data are expressed as means ± s.e.m. and are representative of at least two independent experiments. * p<0.05 and ** p<0.01 (Hsp70 immunized WT mice compared to control WT mice by Mann Whitney U test).

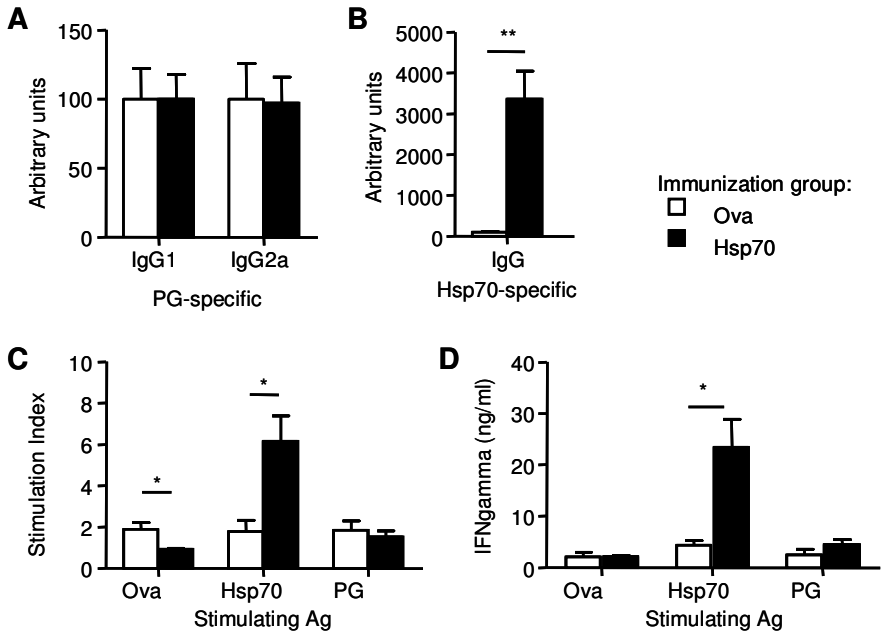

Figure 7 Protective effects found upon Hsp70 immunization are IL-10 dependent. IL10$^{-/-}$ mice were immunized with Ova or Hsp70, subsequently arthritis was induced and scored as described in figure 1. (A) PG-specific IgG1 and IgG2a and (B) Hsp70-specific total IgG production was measured by ELISA in sera obtained on day 52. Data in A and B are depicted relative to a standard of pooled sera from either arthritic mice or Hsp70 immunized mice. (C) Antigen-specific proliferation of spleen cells harvested on day 52 was measured after 72 hours culturing in the presence of recombinant microbial Hsp70, Ova or PG. Proliferation was measured by detection of ^{3}H incorporation and depicted as stimulation index (cpm Antigen/cpm background). The mean background values (responses without antigen) were for the PBS control group 1705 ± 339 and for the Hsp70 group 1394 ± 468. (D) After 72 hours incubation, antigen-induced IFN-γ excretion in the culture supernatants was analyzed by ELISA. Values represent means ± s.e.m. (n=5 mice in each group). Significant difference (p<0.05, Mann-Whitney U Test) is indicated by the asterisk.

Discussion

The anti-inflammatory properties of Hsp have been shown in several studies in both animal models and in patients suffering from inflammatory diseases (1, 5, 11, 12, 23, 24). However, for further development of Hsp for therapeutic application, it is essential to understand the mechanisms by which Hsp affect chronic inflammatory disease in more detail. In this study we analyzed the protective potential of Hsp70 in PGIA, a chronic model for RA, studied the T cell response induced by Hsp70 immunization and addressed whether the presence of IL-10 was essential. Our data clearly show that pretreatment with Hsp70 delayed arthritis onset and dramatically reduced disease severity both clinically and histologically. Furthermore, it increased the antigen-specific T cell response to both the protective Hsp70 and the disease inducing PG. In IL-10 deficient mice Hsp70 immunization did not suppress arthritis illustrating the IL-10 dependency of Hsp70 induced immunoregulation.

The immunoregulatory effect of Hsp70 immunization has been demonstrated earlier in arthritis models in rats (9, 25, 26). In contrast to the rat models, the mouse PGIA model is a chronic and progressive model of RA (27). In addition, in this model arthritis can be induced by immunization with proteoglycan mixed with synthetic adjuvant DDA instead of CFA (18). Therefore, the possibility of interfering immune responses induced by mycobacterial Hsp present in CFA can be excluded.

In the present study an Hsp70-specific T cell response was induced upon Hsp70 immunization as detected by increased proliferation, IL-10 and IFN-γ production upon Hsp70 *in vitro* restimulation. Remarkably, also an enhanced PG-specific T cell response, with a similar cytokine profile, was found in Hsp70 immunized mice. This might be explained by Hsp70 interaction with innate receptors on antigen presenting cells, resulting in enhanced antigen priming to the subsequent PG immunization. Similarly, peptide-specific $CD4^+$ T cell proliferation has been described to be enhanced when immunogenic peptides were fused to Hsp70 subsequently resulting in increased immunogenicity of the peptides (28). Alternatively, Hsp70-induced regulatory T cells might induce a tolerogenic micro-milieu, by their cytokine production, enhancing the induction of regulatory T cells via infectious tolerance. This has been shown to occur in other models of autoimmunity in an IL-10 dependent manner (29).

Our data show that Hsp70 immunization induces a population of IL-10 producing T cells that do not express enhanced levels of Foxp3. Besides Foxp3 expressing $CD25^+$ T cells, additional regulatory T cell subsets, such as adaptive Tr1, that do not express high levels of Foxp3 constitutively, are known (30). Tr1 cells are induced in the periphery upon encounter of antigen and express high levels of IL-10 (31). The Hsp70-specifc IL-10 production together with enhanced IL-10 mRNA expression in $CD4^+CD25^+$ T cells in Hsp70 immunized

mice suggests activation or induction of Hsp70-specific regulatory T cells. However, our observation that also $CD3^-$ cells produce enhanced levels of IL-10 in Hsp70 immunized mice (data not shown) indicates that multiple subsets of regulatory cells can contribute to regulation and this remains to be elucidated. This is in contrast to a recent study, where we showed an increase in Foxp3 expressing regulatory T cells, after Hsp treatment, in a mouse atherosclerosis model (4). However, in the present study $CD4^+CD25^+$ T cells from Hsp70 immunized mice did not increasingly express Foxp3, indicating the possible presence of distinct types of regulation in different models of inflammation.

B cell responses play an important role in the pathogeneses of RA as demonstrated by the success of B cell targeted therapy, for example with monoclonal antibodies like Rituximab, as recently summarized (32). Also in the PGIA model, B cells are important for disease induction (17, 33). Therefore, the effect of Hsp70 immunization on the B cell response was addressed by measuring PG-specific IgG1 and IgG2a production. Interestingly, decreased PG-specific IgG2a production was found in Hsp70 immunized mice, while the IgG1 levels were not significantly changed. In earlier studies the IgG1 isotype has been shown to be the dominant isotype in the PGIA model. However, in agreement with our data, IgG2a levels have been described to correlate with disease severity (34). Furthermore, the IgG2a isotype is known to be Th1 related. In accordance with these findings PGIA is considered to be a Th1 mediated model. Thus our data indicate that the pathogenic Th1 mediated PG-specific antibody response was inhibited by Hsp70 immunization. In contrast to the arthritis associated PG-specific antibody response, Hsp-specific antibodies have been shown to suppress adjuvant induced arthritis in rats (21, 22). Therefore, the increased Hsp70 IgG production found in our study in Hsp70 immunized mice might be important for the regulatory potential of Hsp70 treatment.

The increased Hsp70-specifc B and T cell response, found in WT mice after Hsp70 immunization, was also found in IL-$10^{-/-}$ mice. However, in the absence of IL-10 this response failed to suppress the pathogenic PG-specific B cell response and it did not increase the PG-specific T cell response as observed in WT mice. So, despite the induction of an obvious Hsp70-specific response, the protective effects of Hsp70 immunization on the PG-specific B and T cell response were shown to be IL-10 dependent, consequently leading to development of severe arthritis in the absence of IL-10. The important role of IL-10 in arthritis has been studied previously in the PGIA model. In this study transfer of cells expressing IL-10 after retroviral IL-10 gene transduction could suppress arthritis (35). Moreover, IL-10 has been described to stimulate PG synthesis and to reverse cartilage degradation induced by activated mononuclear cells (14) and it negatively correlated with progression of joint destruction in RA (36), showing the important anti-inflammatory function of IL-10 in human RA.

Due to their stress-inducible nature Hsp can be ideal candidates for immunotherapy against chronic inflammatory diseases like RA. Hsp will be up-regulated, locally at the site of inflammation in the joint, as reported previously (37-39). Therefore, Hsp-specific immune responses can be targeted specifically to sites of inflammation. Moreover, enhanced Hsp-specific T cell responses in patients have been described (5, 6, 40). The observation that such responses seemed to be associated with a benign form of disease, which over time preceded disease remission (5, 6), and the fact that Hsp-mediated preventive and therapeutic immune interventions were effective in animal models of chronic inflammatory diseases, suggests the immunotherapeutic potential of Hsp in patients with inflammatory autoimmune diseases.

In summary, this study shows that Hsp70 can modulate inflammation in a model for chronic and progressive arthritis through IL-10 dependent mechanisms, operating via suppression of pathogenic antibody responses and the development of regulatory T cells. The chronic and progressive nature of the model and the possibility to use specific knock out mice, like the $IL10^{-/-}$ used in this study, will enable further mechanistic studies needed for the development of Hsp based immune intervention in chronic inflammatory diseases.

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CHAPTER 5

Oral or nasal antigen induces regulatory T cells that suppress arthritis and proliferation of arthritogenic T cells in joint draining lymph nodes

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Abstract

The propagation of mucosal tolerance in autoimmune diseases remains a difficult goal to achieve, therefore further mechanistic studies are necessary to develop potential clinical protocols to induce mucosal regulatory T (Tr) cells. In this study we addressed oral or nasal proteoglycan induced functional Tr cells in the cartilage proteoglycan induced chronic arthritis model. Both nasal and oral application of human proteoglycan before induction of disease suppressed arthritis severity and incidence. Tolerized mice showed enhanced numbers of IL-10 producing $CD4^+$ cells in the paw-draining lymph nodes. Furthermore, $CD4^+$ spleen cells displayed enhanced expression of molecules associated with Tr cells, such as IL-10, Foxp3 and TGF-β. Transfer of $CD4^+$ spleen cells from mucosally tolerized donors into proteoglycan-immunized mice abolished arthritis and reduced humoral responses, indicative of Tr cells with the capacity to inhibit already induced immune responses. Tr cells were activated upon transfer, because enhanced proliferation was observed in the joint draining lymph nodes compared to activated T cells from non-tolerized donors. Upon cotransfer with naive proteoglycan-specific T cells, mucosally induced Tr cells inhibited proliferation of these arthritogenic T cells in vivo. Herein we show that both oral and nasal Ag application induced Tr cells, which had a direct inhibitory effect on already established pathogenic B and T cell responses.

Introduction

Mucosal tolerance is important to prevent inadequate and pathogenic reactivity to normally innocuous antigens that enter the body via mucosal surfaces. In animal models of inflammatory diseases, both oral and nasal antigen application has been shown to protect against the induction of disease via the induction of regulatory T cells (Tr cells) (1, 2). Mucosal tolerance has been proposed as a means to induce antigen-specific protection in inflammatory diseases, ranging from autoimmune disease to transplant rejection and allergy (3, 4). However, successful translation of prevention of disease in animal models to therapeutic application in human disease proved to be difficult (5-11). The disappointing outcome of clinical trials can be explained by several facts, such as: these trials were performed in end-stages of disease; the patient populations studied were inherently diverse; and a lack of pretreatment screening to determine whether the antigen was immunologically relevant in patients to be treated.

Despite these difficulties, it is unequivocal that oral tolerance can be achieved in humans (12-17). In some cases, antigen fed suppressed *in vitro* T cell recall responses, but failed to suppress antigen-specific IgG, IgM and secretory IgA responses, suggesting a differential effect on T and B cell responses (12). Intriguingly, this seems not be the case in IgE-mediated allergy, as it was shown that desensitization by oral immunotherapy was highly successful (18).

Previously, it was demonstrated that relevant epitopes of the cartilage proteoglycan were recognized by T cells in the context of human class II MHC in HLA-DR4- and HLA-DQ8-Tg mice (19). Moreover, several studies in rheumatoid arthritis patients showed T and B cell responses against the human proteoglycan, indicating that PG may be a potential auto-antigen in RA. (20-22). Collectively, these studies suggest that PG may be a target of disease-associated T cell responses in patients with RA (23-26) (Berlo et. al. unpublished results). Difficulties with translating animal models into clinical trials clearly show the need to further unravel the complex mechanisms of mucosal tolerance, and to explore their application in experimental autoimmune disease models. Because orally and nasally induced tolerance might induce different Tr cells (27) we wanted to explore both forms of tolerance induction in the PG-induced arthritis model (PGIA). PGIA is a chronic relapsing model for rheumatoid arthritis and disease is based on a combined T and B cell response directed against joint cartilage PG (28-30).

In this model we studied the potential regulatory role of mucosally induced Tr cells and whether these cell could alter the *in vivo* response of potentially arthritogenic T cells.

Oral and nasal application of PG was found to suppress the induction of disease in a comparable fashion. The reduction of arthritis severity correlated with enhanced numbers of IL-10 producing antigen-specific $CD4^+$ T cells in the local draining lymph nodes. Additionally, enhanced mRNA levels for IL-10 and Foxp3 in $CD4^+$ splenocytes indicated the presence of increased numbers of functional Tr cells within this T cell pool, as also shown by reduced

arthritis severity in acceptor mice upon $CD4^+$ spleen cell transfer. Furthermore, transfer of mucosally induced Tr cells led to IL-10 production in the joint draining lymphoid tissues and reduced influx of $CD4^+$ cells and neutrophils into the joints. In the same experiments, transferred (CFSE-labeled) mucosally induced $CD4^+$ Tr cells exhibited enhanced proliferation in the joint draining lymph nodes and inhibited proliferation of naive arthritogenic T cells *in vivo.*

Introduction

Mice

Female BALB/c retired breeder mice, aged between 16-26 wk, were purchased from Charles River Laboratories. PG-TCR transgenic (Tg) mice were bred and kept under standard conditions (31,32). Animals were routinely housed and received water and chow *ad libitum*. Experiments were approved by the Animal Experimental Committee of the University of Utrecht.

Antibodies and antigen

Anti-CD8 (53-672), anti-CD11b (M1/70, MAC-1), anti-F4/80, anti-MHC class II (M5/114) and anti-CD45R (6B2) were used as culture supernatants at predetermined optimal concentrations. PE-conjugated anti-CD4 (GK1.5), anti-CD25 (PC61), anti-CD69 (H1.2F3) anti-CD103 (M290), anti-CD27 (Lg.3A1) were purchased from BD-Pharmingen.
Proteoglycan (PG) (aggrecan) was purified from human articular cartilage by 4 M guanidinium chloride extraction, and depleted of glycosaminoglycan side chains using endo-β-galactosidase (0.22 mU/mg dry weight) and testicular hyaluronidase (5 units/mg dry weight) as described before (13, 14). For intranasal treatment potentially present contaminating endotoxins were removed from PG by a triton X-114 gradient. After this the PG was extensively washed and a *Limulus* amebocyte lysate assay was performed according to manufacturer's protocol to test for endotoxin contamination. Endotoxin levels were < 50 EU/mg.

Tolerance induction and arthritis induction

Mice were either tolerized by 3x 100 µg endotoxin low PG intranasally (i.n.*)* in 10 µl PBS or 3x 1 mg PG via gavage in 200 µl PBS on days -7, -5, -3. Control mice received ovalbumin (OVA) grade V as a control. Subsequently, mice were immunized for induction of arthritis on days 0 and 21. Mice were injected intraperitoneally (i.p.) with 400 µg PG in the synthetic adjuvant dimethyl dioctadecyl ammonium bromide (DDA; 2 mg) in 200 µl solution with PBS.

Assessment of arthritis

Paws of all mice were examined 3 times per week to record abnormalities due to arthritic changes of the joints. The onset and severity of arthritis were determined using a visual scoring system based on swelling and redness of paws in a blinded setup as described previously (28-30). In brief, the degree of joint swelling for each paw (scored ranging from 0 to 4) was used to express a cumulative arthritis score, with a possible maximum severity index of 16 per animal. The first clinical appearance of swelling was recorded as arthritis onset.

Cytokine secretion and cytokine ELISA

The percentage of cytokine-secreting cells, and the amount of cytokine secreted during a 24-h re-stimulation period, was assessed in spleen and draining lymph node (DLN) cells. Single cell suspensions were incubated at $5x10^6$ cells/ml with 50 µg/ml PG (or medium as control) for 24-h. Brefeldin A was added for the last 4-h of culture. Cells were subsequently stained with anti-CD4 (GK1.5), fixed and permeabilized (Cytofix/Cytoperm, BD Biosciences) and stained with anti-IL-10 (JES5-16) and anti-IFNγ (AN-18). Cells were analyzed on a FACSCalibur and data were analyzed with CellQuest software.

In separate 96 wells plates cells were incubated with either 50 µg/ml PG or medium as a control for 72 h and culture supernatants were analyzed using the Luminex 100 system (BD Biosciences) in combination with the LINCOplex assay performed according to the manufacturer's instructions (Linco Research). In brief, antibody-coated microspheres were incubated with standards, controls, and samples (25 µl) in a 96-well microtiter filter plate overnight at 4ºC. After incubation, beads were washed and a mixture containing IL-10-specific and IFN-γ-specific detection antibodies was added. After 30 min of incubation at room temperature, streptavidin-phycoerythrin (PE) was added for an additional 30 min. After a final wash step, the beads were resuspended in buffer, read on the Luminex model 100 instrument (Luminex Corporation, Austin, TX) to determine the concentration of the cytokines of interest and results were analyzed using LMAT software (Luminex Corporation, Austin, TX).

ELISA for PG-specific antibodies

PG-specific antibodies were measured by ELISA as described previously (28, 30, 33). ELISA 96-well plates (Corning) were coated overnight with hPG (0.1 µg protein/well) or native mPG (0.15 µg protein/well), and blocked with 1% fat-free milk in PBS. Sera were applied at increasing dilutions and isotypes of PG-specific antibodies were determined using peroxidase-conjugated mAbs to mouse IgG1 or IgG2a (BD Biosciences) as secondary antibodies (33). Serum antibody levels were calculated relative to a corresponding mouse IgG isotype standards (all from BD Biosciences) or mouse serum immunoglobulin fractions (Sigma-Aldrich) (28, 30, 33).

Transfer to assess regulatory function of T cells

Single cell suspensions from spleens of donor mice were depleted from erythrocytes in ACK-lysis buffer (150 mM NH_4Cl; 1 mM $NaHCO_3$; pH 7.4) and were stained with monoclonal antibodies-specific for CD45R, CD11b, F4/80, MHC class II and CD8. Positive cells were removed with sheep anti-rat-conjugated Dynal beads (Dynal Biotech).

Negative cells, denoted as enriched $CD4^+$ T cells (purity routinely between 85 and 95%), were resuspended in PBS. Per recipient $1x10^6$ cells were transferred at day 20. Mice were immunized at days 0 and 21 for induction of arthritis. For tracking of $CD4^+$ cells we resuspended enriched $CD4^+$ cells in PBS at 10^7 cells/ml and incubated these for 10 minutes

at 37°C with 5,6-carboxy-succinimidyl-fluoresceine-ester (CFSE) (Molecular Probes) at a final concentration of 5 μM to follow their division profiles *in vivo*. CFSE-labeled $CD4^+$ T cells were washed in ice-cold PBS with 2% FCS and resuspended in saline. Each mouse received $1x10^7$ $CD4^+$ CFSE-labeled cells in 100 μl saline by i.v. injection. For tracking of naive $CD4^+$ T cells isolated from PG -TCR Tg mice cells were labeled with PKH26. Cells were incubated with $2x10^{-6}$ M PKH26 dye in diluent C for 5 minutes at 25°C at $2x10^7$ cells/ml. Labeled cells were washed in PBS with 2% FCS and resuspended in saline.

Quantitative PCR

For quantitative analysis of mRNA expression, $1x10^6$ cells or total joint-extracted cells were isolated and total RNA was isolated using the Qiagen RNeasy kit (Westburg, Leusden, The Netherlands). Subsequently, RNA was transcribed into cDNA using the iScript cDNA Synthesis Kit (Biorad) according to the manufacturer's protocol. Real time quantitative PCR (Q-PCR) was performed using a Biorad Icycler based on (specific primers and) general fluorescence detection by SYBR green. HPRT and GAPDH were used as control for sample loading and to allow normalization between samples. cDNA obtained from lymphoid tissues from naive mice was used to allow normalization between experiments. Primers used were: HPRT sense 5'-CTG GTG AAA AGG ACC TCT CG-3, antisense 5'-TGA AGT ACT CAT TAT AGT CAA GGG CA-3'. GAPDH: sense 5'- CAA CTC ACT CAA GAT TGT CAG CAA-3' antisense 5'-GGC ATG GAC TGT GGT CAT GA-3' Foxp3: sense 5'-CCC AGG AAA GAC AGC AAC CTT-3', antisense 5'-TTC TCA CAA CCA GGC CAC TTG-3'. IL-10: sense 5'-GGT TGC CAA GCC TTA TCG GA-3', antisense 5'-ACC TGC TCC ACT GCC TTG CT-3'. CD4: sense 5'- GAC TGA CCC TGA AGC AGG AG-3', antisense 5'-CTG GTT CAC CCC TCT GGA TA-3'. Myeloperoxidase (MPO): sense 5'-GCT ACC CGC TTC TCC TTC TT-3', antisense 5'-GGT TCT TGA TTC GAG GGT CA-3'.

Statistics

Data were analyzed with a one way ANOVA with a Bonferroni post-hoc test to determine significance for differences between individual groups. $P<0.05$ was considered significant.

Results

Mucosal antigen application prevents PGIA

To explore whether mucosal tolerance suppressed disease in the chronic PGIA model, PG was applied on days -7, -5 and –3 either i.n. or intragastically (i.g.). Control mice received OVA protein i.n. and/or i.g. All mice were subsequently immunized for the induction of arthritis on days 0 and 21.

Intranasal and oral application of PG before immunization significantly reduced the development of arthritis as shown by the lower mean arthritis score when compared to control OVA treated mice (Figure 1). Also, the maximum arthritis score of the individual animals was reduced. OVA-treated controls showed an average maximum score of 7.4 ± 4.0, while tolerized animals after nasal treatment only reached a maximum score of 3.2 ± 2.6 and orally tolerized animals of 2.7 ± 2.4 (Table 1).

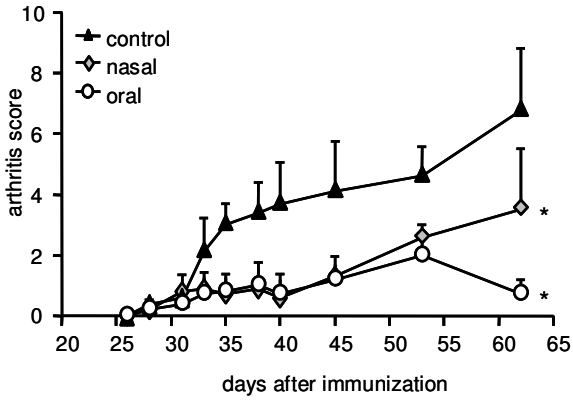

Figure 1. Mucosal application of human PG induces tolerance. All mice were immunized by two immunizations with human PG at day 0 and day 21 to induce arthritis. Before the first immunization mice were tolerized with either 100 µg hPG in 10 µl PBS intranasally (◊) or 1 mg hPG orally (○) at days -6, -3 and -1. Control mice received OVA mucosally. Arthritis scores were analyzed 3 times a week with a maximum score of 16 per mouse. Data are the mean values of at least 5 mice per group ± standard deviation. Data are representative of four separate experiments. * p<0.05 to control.

Table 1. Mean day of onset and arthritis score

	Donor		Acceptor	
	Mean day of onset	Max. arthritis score	Mean day of onset	Max. arthritis score
Control	29.1 ± 5.1	7.4 ± 4.0	26.6 ± 3.5	9.3 ± 3.1
Oral	36.2 ± 7.4 *	2.7 ± 2.4 **	31.7 ± 7.2 *	5.3 ± 2.9 *
Nasal	34.5 ± 6.6 *	3.2 ± 2.6 **	29.7 ± 4.1	5.0 ± 2.0 *

All mice were immunized by two immunizations with 400 µg human PG and 2 mg DDA in 200 µl PBS at day 0 and day 21. Prior to the first immunization, donor mice were tolerized with either 100 µg human PG in 10 µl PBS intranasally (Nasal) or 1 mg PG orally (oral) at days –6, -3, and –1. Control mice received OVA mucosally (control). Acceptor mice received $1x10^6$ CD4+ splenocytes from donor mice at day 20 isolated as describe in materials and methods. * p<0.05 and ** p,0.01 compared to control.

Assessment of the mean day of arthritis onset revealed that mucosally treated mice not only developed less severe disease, but also that initial signs of disease occurred significantly later (Table 1). These data shows that mucosal tolerance is a powerful means to reduce arthritis induction in the PGIA model.

Mucosal antigen enhances Foxp3, IL-10 and TGF-β expression by CD4$^+$ splenocytes

Subsequently we analyzed the effect of mucosally applied antigen on differentiation of CD4$^+$ T cells within the paw draining lymph nodes (DLN) and spleens of mucosally tolerized mice as based on cytokine profile and surface marker expression. DLN and spleen cells were isolated on day 40 after the first immunization and single cell suspensions were cultured for 24 h in the presence of 50 µg/ml PG. The last 4 hours of culture brefeldin A was added to analyze the intracellular cytokine content. For expression of surface markers, cells were analyzed without prior *in vitro* reactivation. Both intranasally and orally tolerized mice showed enhanced numbers of PG-specific cells producing IL-10 within the DLNs compared to OVA-treated controls. Fewer IL-10 producing cells in the OVA treated control mice coincided with enhancement of the IFN-γ producing cell population in the DLN (Figure 2).

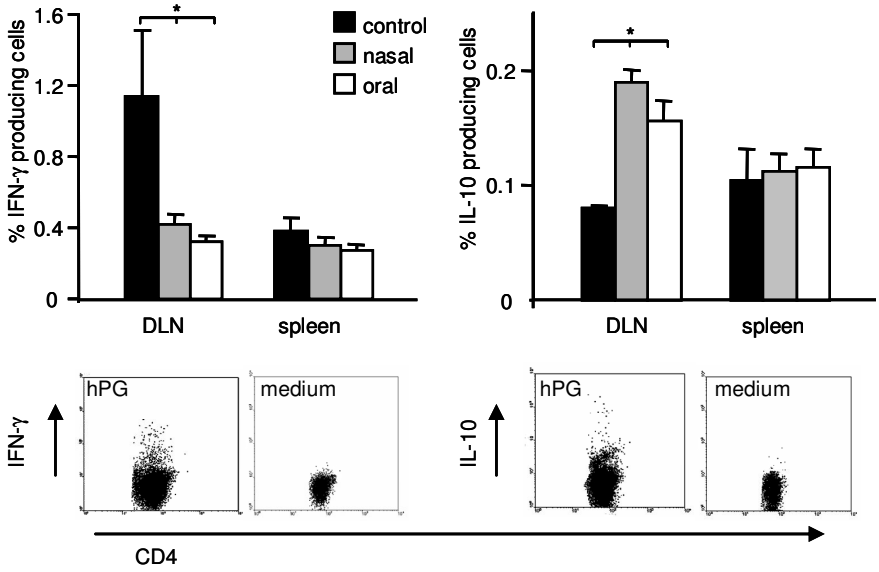

Figure 2. CD4$^+$ splenocytes of tolerized mice produce more IL-10. Mice were treated as described in figure 1. Spleen cells were isolated at day 40 and single-cell suspensions were (re)stimulated *in vitro* for 24 hours with 10 µg PG or medium as control. Brefeldin A was added for the last 5 hours. Subsequently, cells were fixed and stained for intracellular cytokines IFN-γ and IL-10. Samples are represented as net producing cells compared to medium control samples. FACS plots are representative figures for IFN-γ and IL-10 producing populations. Data are the mean values of at least 5 mice per group ± SD. * $p<0.05$ to control. Data are representative of at least three separate experiments.

Several studies have demonstrated that oral antigen application induced Tr cells residing within the $CD4^+$ spleen population. To address whether $CD4^+$ spleen cells obtained a regulatory phenotype in PGIA, cytokine expression within this population was assessed. Within the spleen cell population of the protected mice, only few cells were found to produce IL-10 in an antigen-specific manner. Therefore, no differences as found in DLN can be observed by flow cytometry. However, when $CD4^+$ cells were isolated to assess *in situ* expression of IL-10, TGF-β and Foxp3 (Figure 3), in both nasally and orally tolerized mice the levels of IL-10, TGF-β and Foxp3 mRNA were enhanced in the splenic $CD4^+$ population compared to control treated arthritic mice. The observed differences in cytokine expression did not correlate with differences in surface marker expression of CD25, CD103 or CD27 within the $CD4^+$ population as measured by flow cytometry in both spleen and DLN derived cells (data not shown). In sum, these data showed that PG-specific T cells in the DLN of tolerized mice displayed a regulatory cytokine profile upon antigen recognition. In addition splenocytes of these mice showed enhanced transcription of IL-10, Foxp3 and TGF-β, all coding for proteins associated with immunomodulatory Tr cells.

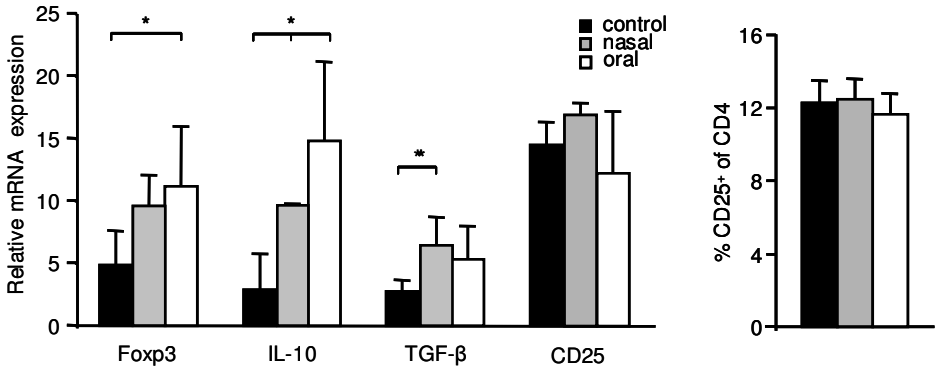

Figure 3. $CD4^+$ splenocytes of tolerized mice express higher levels of Foxp3, IL-10 and TGF-β mRNA. Mice were treated as described in figure 1. Spleen cells were isolated at day 40 and mRNA was isolated directly. cDNA was used in Q-PCR/SYBR green analysis for Foxp3, IL-10, TGF-β and CD25. Data are expressed as relative expression to a calibrator ± s.e.m. of at least 5 mice per group and are representative of at least three separate experiments.

Mucosal antigen induces $CD4^+$ Tr in the spleen with the capacity to suppress arthritis

To determine whether the enhanced expression of regulatory molecules observed in spleen reflected functional differentiation of mucosal Tr cells, we isolated $CD4^+$ splenocytes from intranasally tolerized, orally tolerized and OVA treated control mice. These $CD4^+$ splenocytes, potentially containing Tr cells, were subsequently transferred i.v. to mice on day 20, before the second PG immunization on day 21. As shown in figure 4, mice that received $CD4^+$ splenocytes from nasally or orally tolerized mice only developed mild arthritis

compared to control groups, indicating that $CD4^+$ spleen cells from mucosally tolerized mice had obtained regulatory capacity. On the other hand $CD4^+$ splenocytes from OVA tolerized control mice slightly enhanced the onset of arthritis as several mice already showed signs of arthritis at day 26 whereas donor mice developed the first clinical arthritic scores on average at day 29 (Table 1). Next to a significant reduction of the clinical arthritis course also the maximum arthritis score was reduced when mice received $1x10^6$ $CD4^+$ splenocytes from orally or intra-nasally treated animals (Table 1). Thus, oral or nasal application of PG prior to induction of arthritis seemed to have induced functional Tr cells that suppressed arthritis in already immunized acceptor mice.

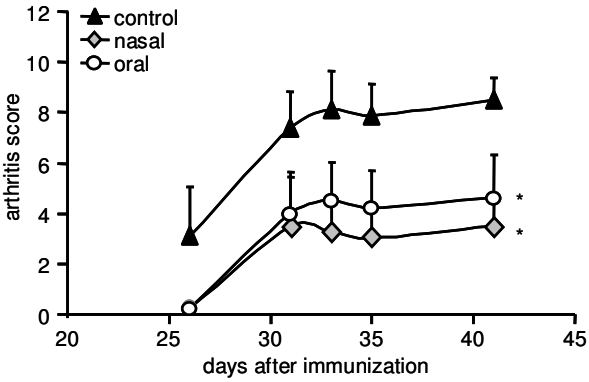

Figure 4. $CD4^+$ splenocytes transfer tolerance to arthritogenic immunization in recipient mice. All mice were immunized with 400 µg hPG and 2 mg DDA in 200 µl PBS at day 0 and day 21. Before the second immunization, mice received $1x10^6$ $CD4^+$ splenocytes from mice that were tolerized as described in figure 1. Arthritis scores were analyzed 3 times a week (with a maximum score of 16 per mouse Data are the mean values of at least 5 mice per group ± standard deviation. Figure is representative of three separate experiments. *, $p<0.05$ to control.

Transferred mucosal Tr cells suppress both T and B cell immunity in arthritis

Since PG-specific auto-antibodies are essential for inducing severe arthritis in PGIA, the effect of Tr cell transfer on the B cell response was studied. Both nasally and orally induced Tr cells suppressed the B cell dependent antibody response as measured by the levels of antigen-specific IgG1 and IgG2a (Figure 5). Transfer of either orally or nasally induced Tr cells significantly reduced the Th1 mediated PG-specific IgG2a antibody levels in serum. Not only was the IgG2a response against immunizing human proteoglycan reduced, also the mouse-PG-specific IgG2a response was significantly reduced upon transfer of Tr cells. In contrast, PG-specific IgG1 antibody levels were only significantly reduced by transfer of orally induced Tr cells. In summary, transfer of both nasally and orally induced Tr cells suppressed human and mouse PG-specific B cell responses. To follow the effect of Tr cell transfer on infiltration of T cells and neutrophils into the joints of arthritic Tr cell recipients, we analyzed CD4 and MPO contents in joint infiltrates. Q-PCR analysis of cells isolated from the joints of Tr cell recipients showed a clear reduction of infiltrating $CD4^+$ and MPO^+ positive cells, indicating reduced influx of both $CD4^+$ T cells and neutrophils (Figure 6). This observation correlated with the reduced arthritis scores as shown in figure 4.

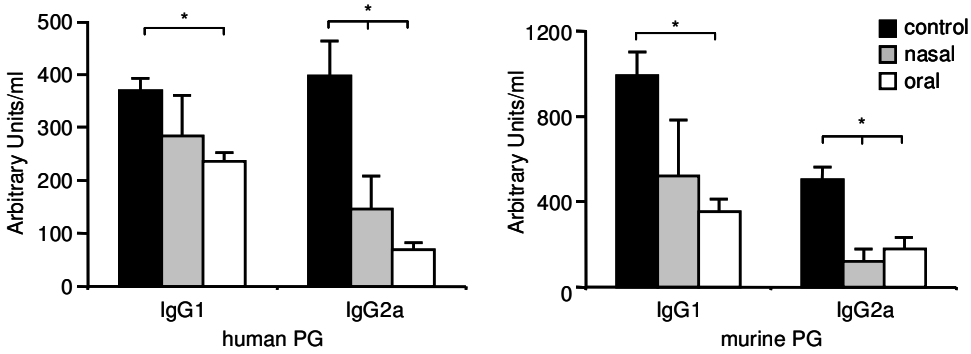

Figure 5. Reduced antibody response to human PG and murine PG after transfer of Tr cells. Mice were treated as described in figure 4. and at day 40 blood was drawn and serum was analyzed by ELISA for IgG1 and IgG2a to human and mouse proteoglycan. Data are the mean values of at least 5 mice per group ± standard deviation.

Tr cell acceptor mice show enhanced numbers of IL-10 producing T cell within the joint draining lymph nodes

To analyze the immunomodulatory role of the transferred Tr cells in recipient mice, we analyzed the proteoglycan-specific cytokine response in the joint draining lymph nodes. Single-cell suspensions of the paw draining lymph nodes were restimulated at $5x10^6$ cells/ml with 50 µg/ml PG *in vitro* during 18 h and brefeldin A was added for the last 6 h. For the analysis of cytokine secretion in the culture supernatant, cells were stimulated for 72 h in the absence of Brefeldin A. Transfer of nasally and orally induced Tr cells enhanced not only the percentage of IL-10 producing $CD4^+$ T cells (Figure 7A), but also significantly enhanced the concentration of IL-10 in response to PG in the culture supernatant (Figure 7C). Additionally, PG-specific IFN-γ secretion was significantly reduced in Tr cell recipients when compared to mice receiving control cells (Figure 7B). In contrast, no significant differences were detected in the concentration of TNF-α (Figure 7D) or the relative expression of IL-17 mRNA (Figure 7G).

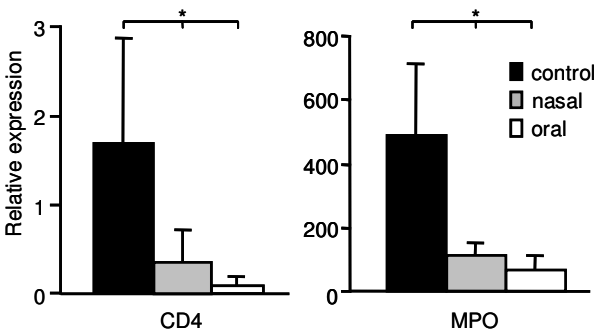

Figure 6: Reduced infiltration of CD4 T cells in joint tissue of Tr cell acceptor mice. Mice were treated as described in Figure 4. At day 40 cells were isolated from the knees of arthritic mice. mRNA was isolated, subsequently treated with DNAse and reversely transcribed to cDNA. Q-PCR analysis using cDNA was performed for CD4 and MPO to assess infiltration of $CD4^+$ T cells and neutrophils. mRNA is normalized to HPRT as housekeeping gene. Mean ± SD.

Also, spleen cells from Tr cell recipients restimulated with PG *in vitro* produced significantly more IL-10 than cells from control animals did (data not shown). Paw draining lymph node cells showed significantly enhanced expression of IL-10 mRNA *in situ* in mice that received intra-nasally induced Tr cells compared to lymph nodes from controls (Figure 7E). Also recipients of orally induced Tr cells showed enhanced relative expression of IL-10, although this effect was not statistically significant. Additionally, the Foxp3 expression in the draining lymph nodes of mice that received orally induced Tr cells was enhanced (Figure 7F). These data indicate that transfer of Tr cells from mucosally tolerized mice modulated the cytokine profile in PG-specific T cells in the paw draining lymph nodes as shown by the reduced IFN-γ production.

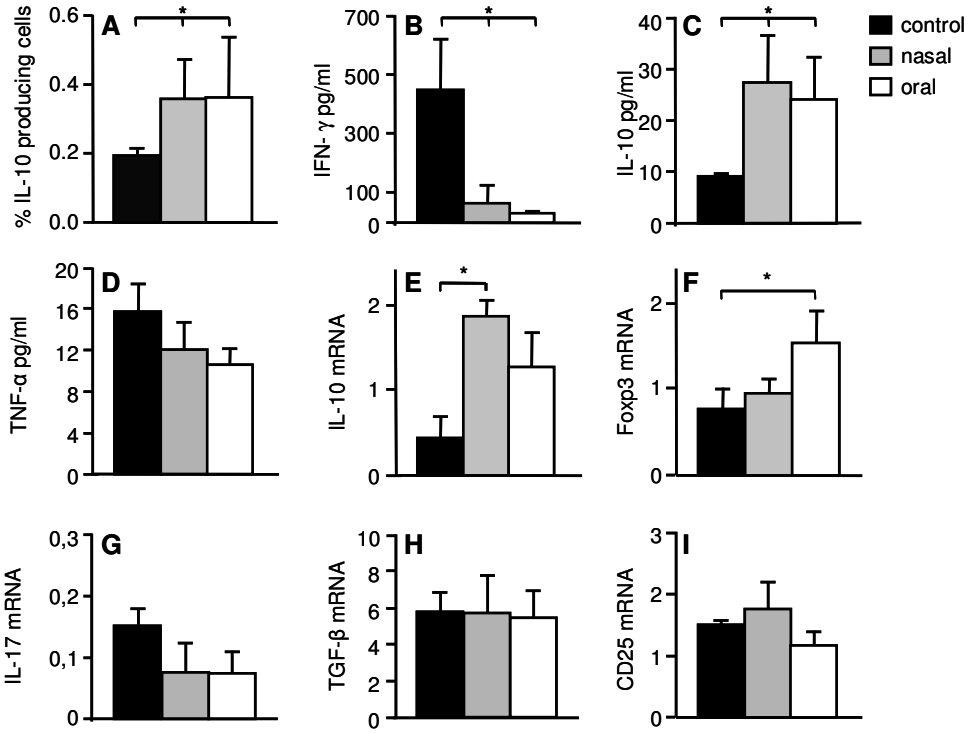

Figure 7: Enhanced numbers of IL-10 producing cells and IL-10 secretion upon transfer of Tr cells. Mice were treated as described in figure 4. (A) Intracellular staining for IL-10. Draining lymph node cells were isolated at day 40 and single cell suspensions were restimulated *in vitro* for 18 h with 10 µg/ml PG. Brefeldin A was added for the last 4 h. Samples are represented as net producing cells compared to medium control samples. (B-D) Single cell suspensions were restimulated with 50 µg/ml human PG and supernatants were analyzed after 72 hours. (E-I) Lymph node cells were isolated at day 40 and mRNA was isolated directly. cDNA was used as template for Q-PCR analysis for IL-10, Foxp3, IL17, TGF-β and CD25. Data are expressed as relative expression to a calibrator. Data are respresented as average ± s.e.m. * $p<0.05$ to control

Tr cells proliferate in the DLN and inhibit proliferation of naive T cells

Since it is currently unclear where transferred Tr cells exert their regulatory function, we aimed to identify the location of Tr cell activation. Therefore, we labeled $5x10^6$ $CD4^+$ mucosally induced Tr cells with CFSE prior to transfer into already immunized recipient mice. Subsequently, mice were immunized the next day and spleen and paw draining lymph nodes were isolated 4 days after the second PG immunization and were characterized for proliferation by flow cytometry of CFSE dilution. Comparable numbers of CFSE labeled cells were detected in spleens and all lymph nodes analyzed of recipient mice irrespective of the tolerization route of the donor mice, indicating that cells migrated through the lymphoid tissues equally well. Intriguingly, we observed significantly enhanced proliferation of transferred Tr cells within the paw draining lymph nodes as compared to control cells (Figure 8 left panel).

Co-transfer of CFSE labeled Tr cells and naive PG-specific TCR-transgenic $CD4^+$ T cells labeled with PKH-26 enabled us to distinguish between division of Tr cells and (potentially) arthritogenic T cells. Although most PKH-26 labeled cells proliferated within the first 5 days after immunization with their cognate antigen proteoglycan, a significant suppression of proliferation was observed in the DLN of mice that received Tr cells. This indicated that mucosally induced $CD4^+$ Tr cells had the capacity to suppress the expansion of arthritogenic T cells *in vivo* (Figure 8 right panel).

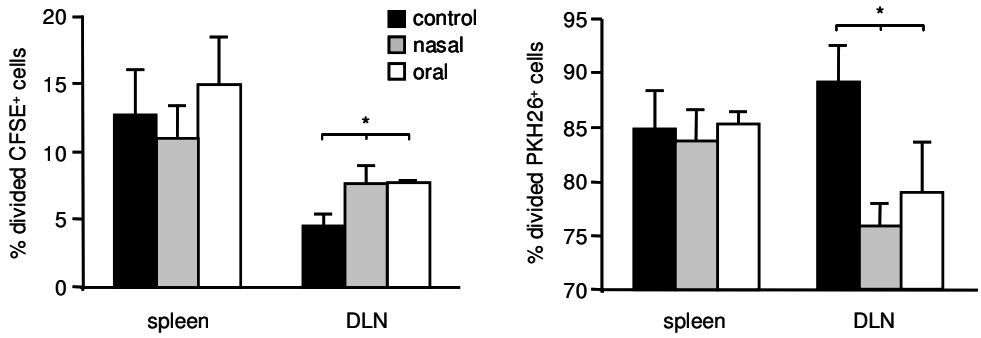

Figure 8. Transferred Tr cells show enhanced proliferation in DLN. $CD4^+$ splenocytes from mucosally tolerized donors were labeled with CFSE before transfer. Naive $CD4^+$ splenocytes from PG-TCR Tg mice were labeled with PKH26 and transferred as described in materials and methods. Five days after transfer into PG-immunized acceptor mice spleen and draining lymph node cells were isolated and analyzed for CFSE dilution. The percentage of $CFSE^+$ cells that had divided is plotted. * $p<0.05$ to control. Data are the mean values of at least 5 mice per group ± SD.

Discussion

Mucosal administration of auto antigens has been shown to be a powerful way to induce tolerance in several models of autoimmune inflammation. However, trials of antigen feeding in patients with chronic autoimmune diseases have been disappointing. Therefore, unraveling the cellular basis of antigen-specific mucosal tolerance in more detail may help clinical application of mucosal tolerance induction. In this study we show the induction of mucosal tolerance in PGIA, a model for chronic and progressive arthritis that crucially depends on T and B cell mediated responses. The clinical relevance of this antigen is growing since literature shows that at least a subset of patients with RA exhibits antigen-specific T and B cell responses against cartilage matrix components (20-22).

This is the first study showing that both nasal and oral application of proteoglycan can suppress PGIA via the induction of mucosal Tr cells. Both routes of antigen delivery significantly reduced arthritis severity and incidence and altered the T cell response in the joint draining lymph nodes. Earlier studies in the laboratory showed that treatment of mice with either soy bean trypsin inhibitor or mouse serum albumin did not induce protection against arthritis (maximum score ± s.e.m. of control group 3.3 ± 0.8 vs mouse serum albumin treated mice 4.3 ± 1.8), underlining the antigen specificity of the response. In addition, adoptive transfer of $CD4^+$ T cells from mucosally tolerized mice showed that functional Tr cells had developed with the potential to suppress T cell and B cell mediated immunity in already immunized mice. These transfer studies also showed that Tr cells isolated from spleen migrated to the joint draining lymph nodes and suppressed proliferation of naive PG-specific T cells *in vivo* under disease inducing conditions.

Most studies exploring the role of mucosal tolerance in autoimmune diseases have focused on oral tolerance induction. However, for practical reasons the nasal route might be more attractive as therapeutic approach compared to oral application. Nasal application will most likely lead to reduced antigenic degradation thereby lowering the dose of antigen needed to achieve tolerance. As we have demonstrated previously, the conversion of naive T cells into functional mucosal Tr occurs in the mucosa-draining lymphoid tissue within 48-72h after antigen encounter and the dose needed for the induction of nasally induced Tr cells was 175 times lower than the dose needed for the induction of oral Tr cells (34-36).

Our data confirm earlier findings on nasal tolerance in arthritis (37-39). However, our study is the first showing that nasal application of PG was sufficient to induce functional Tr cells that are suppressive upon transfer without further treatment. This is in contrast with an earlier study in the PGIA model (33) which showed that continuous nasal treatment was needed to maintain tolerance after transfer of splenocytes of tolerized mice to SCID acceptor mice. This difference can be explained by a difference in Tr cell population, since we transferred $CD4^+$ T cells to immune competent hosts instead of splenocytes to SCID mice. In the earlier study transfer of unfractioned spleen populations may have led to co-transfer of potentially arthritogenic cells that contributed to disease induction.

Mucosal tolerance to antigens has been considered an effective means to prevent T cell mediated immune responses to the same antigen. In humans however, oral tolerance failed to suppress antigen-specific B cell responses (12). B cells are known to play a crucial role in the pathogenesis of RA, via the induction of auto-antibodies, activation of auto-reactive T cells and formation of tertiary lymphoid structures (40). Modulation of the B cell response via mucosal tolerance induction could therefore strongly enhance therapeutic benefit. Herein we show that mucosal Tr cells are capable of suppressing the Th1 mediated antigen-specific IgG2a response irrespective of the site of their induction, to both the tolerizing antigen and the murine PG. However, only orally induced Tr cells also suppress the antigen-specific IgG1 response, indicating a more general suppression of both Th1 and Th2 cell mediated immune responses. This is in agreement with an earlier study showing that orally tolerized T cells can no longer provide cognate help to B cells (41). Given our finding that nasal tolerance was effectively suppressing disease, we may conclude that suppression of IgG1 is not essential for suppression of disease. These findings indicate that although both oral and nasal tolerance can induce tolerance via the induction of Tr cells the suppressive mechanisms might differ.

Even though both oral and nasal antigen application resulted in suppression of disease no obvious changes in cell surface marker expression of T cells in draining lymph nodes or the spleen of treated mice were detected. This is in agreement with recent studies exploring the phenotype of mucosally induced Tr cells rapidly after antigen application, as these studies showed that such Tr cells can hardly be distinguished from other recently activated T cells and show that regulatory capacity resides in both $CD25^+$ and the $CD25^-$ populations (34-36). In addition, phenotypic differences in a small population will not be reflected by differences in the entire $CD4^+$ T cell population in spleen. However, the observation that differences in regulatory markers are present in spleen mRNA in combination with their ability to transfer tolerance to immunized recipients suggests that these Tr cells do reside in this tissue.

In this study we collected evidence that mucosally induced Tr cells were not only able to migrate into the joint draining lymph node, but also that they were activated locally, as clearly shown by their *in vivo* proliferation. Transferred $CD4^+$ cells from non-tolerized mice proliferated significantly less in the DLN, however, we can not exclude that this reduced proliferation compared to Tr cells is due to the activation that occurred in the the severely diseased donor mice. The data indicate that mucosal Tr cells suppressed arthritis development by suppressing proliferation of pathogenic T cells in the joint draining lymph nodes, thereby reducing subsequent immune responses at both the T cell and B cell level, in line with the findings of enhanced IL-10 expression in $CD4^+$ T cells and reduced IgG2a levels.

Herein we show that both oral and nasal antigen application can induce functional Tr cells in the chronic and relapsing PGIA model. It is possible that effective translation into therapeutic application in humans will lead to combination therapy with other anti-inflammatory approaches, such as α-TNFα. Such combination of therapies will then broadly target autoaggressive T effector cells, while inducing or expanding antigen-specific Tr cells,

diverting the autoimmune response into a more regulatory type (42). Recently, the effectiveness of such a combined approach was demonstrated in RA patients (43, 44). The skewing of antigen-specific inflammatory responses toward more tolerogenic responses can become a major addition to available therapeutic options for autoimmune diseases.

Acknowledgements

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CHAPTER 6

Epitope-specific targeting of anti-inflammatory T cell responses with conserved Hsp70 peptides in autoimmune disease

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Abstract

Hsp70 is abundantly expressed by cells during inflammation. Stress enhanced presentation of Hsp70-derived peptides leads to activation of anti-inflammatory T cells. Therefore, we hypothesized that T cell epitopes of Hsp70-derived peptides can be targets for epitope-specific immunotherapy in inflammatory diseases. By epitope mapping, we identified the highly conserved C1 peptide as a dominant T cell epitope of *Mycobacterium tuberculosis* (Mt) Hsp70 in BALB/c mice. C1-specific T cells were cross-reactive with the mouse homologue peptides, mC1a and mC1b. Using a $CD4^+$ T cell hybridoma recognizing mC1b we showed that up-regulation of endogenous Hsp70 in antigen presenting cells enhanced T cell activation. This emphasized that the mC1b peptide was processed and presented from the endogenous Hsp70 under physiological conditions. Nasal application of C1 and its mouse homologues suppressed proteoglycan induced arthritis in BALB/c mice, demonstrating the immunoregulatory potential of the peptides. Furthermore, (m)C1-specific $CD4^+$ T cell responses were found in humans, both in healthy controls and in the inflamed joint illustrating the relevance for clinical application in humans. Since (m)C1 peptides were recognized by human $CD4^+$ T cells it is attractive to speculate that these peptides can be used to amplify the naturally existing Hsp-specific regulatory T cell response in patients with chronic inflammatory disease.

Introduction

Breakdown of peripheral tolerance mechanisms can lead to autoimmune disease, characterised by exaggerated responses of the immune system to self-tissue antigens (1). Between the various autoimmune diseases, basic features of failing regulatory systems and chronic inflammation are shared characteristics (2). Many existing therapies in autoimmune disease are based on general suppression of inflammation and the side effects observed with these therapies illustrate the pressing need for more specific intervention (3, 4). Regulatory T cells (Treg) are pivotal controllers of peripheral tolerance and thus frequently studied targets for immunotherapy (1, 5). Epitope-specific targeting of Tregs would enable precise and site-specific immunomodulation (6, 7) and has proven successful in multiple experimental models of inflammatory disease. In these models, tolerance has been induced with auto-antigens that were frequently also the disease inducing antigen (8). In humans however, disease development is often a multifactorial process and the actual triggers and specific auto-antigens are frequently elusive, which complicates immunotherapy. For that reason peptides of self-antigens that already participate in control of inflammation can be an interesting alternative and we suggested previously that heat shock proteins (Hsp) can be such antigens (4).

The Hsp families consist of multiple members classified by their molecular weight, such as the Hsp60, Hsp70 and Hsp90 families (9, 10). Hsp are highly conserved proteins (11), that can trigger both innate and adaptive arms of the immune system and the final immune response to Hsp can be pro- or anti-inflammatory (12, 13). According to the immunological homunculus autoreactive B and T cell responses and innate receptors for a few dominant self proteins are being used to sense the state of the body in order to select the appropriate immune response (14, 15). As immunodominant antigens, Hsp have been postulated to be part of the immunological homunculus and thus to be involved in control of inflammation.
Besides the fact that Hsp are participating in control of inflammation, several features of Hsp make them attractive candidate antigens for epitope-specific immunotherapy. Hsp expression is enhanced when cells are in danger (10, 16, 17). Thus in response to inflammatory stress Hsp will become highly expressed at the site of inflammation, enabling site-specific activation of antigen-specific Treg. Such increased expression of Hsp has been shown previously in the inflamed synovium in rheumatoid arthritis (RA), and juvenile idiopathic arthritis (JIA) patients and in the inflamed muscle in juvenile dermatomyositis patients (JDM) (18-20). In addition, Hsp70 peptides have been frequently eluted from MHC class II (21-23) [and Supplementary Table 1] illustrating that these self-peptides can be presented to $CD4^+$ T cells. In human JIA patients, T cell responses to especially conserved Hsp60 peptides have been shown to correlate with a favorable course of the disease (24-26). In rodents, self- Hsp-specific T cells have been reported that cross-recognize bacterial-Hsp (27, 28). Furthermore, in multiple experimental models of inflammatory disease

administration of both bacterial- and self-Hsp or Hsp-derived peptides have been shown to suppress disease (29), while this protective response has not been observed with other highly conserved proteins (30). Also in humans, initial clinical trials with Hsp-derived peptides have been successful (31, 32).
Among the family members, the protective effects of Hsp60 have been studied most extensively. However, for multiple reasons Hsp70 is also an interesting candidate. Hsp70 (HspA1A/HspA1B) is one of the best stress inducible Hsp (9) and stress enhanced presence of Hsp70-peptides on MHC class II has been reported (22). The immunosuppressive potential of Hsp70 has been shown in several studies in rodent models (28, 33-35). Nevertheless, studies on the immmunomodulatory capacity of Hsp70, Hsp70-derived peptides and especially the mechanisms, are limited. Furthermore, Hsp70 peptides with immunomodulatory properties, that might be relevant for humans, have not been tested thus far.

In the present study we explored the T cell epitopes of Hsp70 involved in Hsp mediated immunoregulation. We identified the highly conserved C1 peptide as a dominant T cell epitope of Mt Hsp70 in BALB/c mice and showed that peptide-specific T cells were cross-reactive and able to respond to enhanced endogenous Hsp expression. The immunoregulatory capacity of the peptide was shown by suppression of proteoglycan induced arthritis (PGIA). This and the fact that peptide-specific T cell responses were observed in humans, both in healthy controls and in the inflamed joint, make C1 a promising candidate for epitope-specific therapy of chronic inflammatory diseases.

Materials and Methods

Mice

Female BALB/c mice, retired breeders aged between 16-26 weeks (arthritis experiments) or mice aged between 9-12 weeks (all other experiments) were purchased from Charles River (Maastricht, The Netherlands) and housed and fed under standard conditions. Experiments were approved by the Animal Experiment Committee of Utrecht University.

Antigens and peptides

Recombinant Hsp70 of *Mycobacterium tuberculosis* (Mt) was obtained from LIONEX Diagnostics & Therapeutics GmbH (Braunschweig, Germany). To avoid interference of LPS contamination with Hsp70 treatment, Hsp70 containing less than 2.1 EU LPS /mg was used. Ovalbumin (Ova) grade V (Sigma, Zwijndrecht, the Netherlands) and $Ova_{323-339}$ or A5 (adenovirus derived) peptides were used as controls. Overlapping synthetic peptides covering the complete sequence of Mt Hsp70 were prepared by automated simultaneous multiple peptide synthesis (SMPS) as described previously (36). All other peptides were obtained from GenScript Corporation (New Jersey, USA). Peptides were analyzed by reversed phase HPLC and checked via electrospray mass spectrometry on an LCQ ion-trap mass spectrometer (Thermoquest, Breda, The Netherlands). Purity of the peptides ranged between 50 to 90 %.

Identification of dominant T cell epitopes of Mt Hsp70

To identify the dominant T cell epitopes of Mt Hsp70, mice were immunized on day 0 and day 14, by intraperitoneal (i.p) injection of 100 µg recombinant Hsp70 in adjuvant DDA (Sigma), 10 mg ml^{-1} emulsified in PBS in a total volume of 200 µl. At day 28 mice were sacrificed and spleen and lymph node cells were isolated and restimulated with a panel of 123 overlapping 15mer peptides covering the complete sequence of Hsp70, followed by analysis of peptide-specific proliferation as described below. Next, pools of identified dominant peptides were used for immunization. These pools were composed of 4 or 5 peptides (Table 1), based on their degree of sequence identity with mouse Hsp70; Pool A contained non-conserved peptides (peptides A1-4), pool B partially conserved peptides (peptides B1-5) and pool C highly conserved peptides (peptides C1-4). Immunization with 100 µl i.p. and 100 µl subcutaneous in the neck was done at 25 µg ml^{-1} of each peptide and 10 mg ml^{-1} DDA on day 0 and day 14 followed by analysis of peptide-specific proliferation and cytokine production on day 28 as described below.

Nasal administration of Hsp70 or peptides and induction and assessment of arthritis

Mice were pretreated on day -7, -5 and -1 with Mt Hsp70 (30 µg per mouse) or peptides (100 µg per mouse) in 10 µl PBS via intranasal (i.n.) application whereas control mice received 10 µl PBS alone or $Ova_{323-339}$ (100 µg per mouse). Subsequently, arthritis was

induced with PG, prepared as described elsewhere (37), using a standard immunization protocol (38, 39). Briefly, 300 µg PG protein was given i.p with 10 mg/ml of DDA (Sigma,) emulsified in 200 µl PBS on day 0 and 21. After the second PG immunization the paws of mice were examined in a blinded fashion 3 times a week to record arthritic changes of the joints. The onset and severity of arthritis were determined using a visual scoring system based on swelling and redness of paws as previously described (38, 39).

Peptide-specific responses in human PBMC and SFMC.

To study peptide-specific responses in humans, peripheral blood mononuclear cells (PBMC) were obtained from the blood of healthy controls and synovial fluid mononuclear cells (SFMC) from the synovial fluid of one JIA patient that was visiting the outpatient clinic of the departments of Rheumatology & Clinical Immunology and Pediatrics of the University Medical Center Utrecht (Utrecht, The Netherlands) and fulfilled diagnostic criteria of for JIA (40). Cells were isolated by ficoll (Pharmacia, Upsalla, Sweden) density gradient centrifugation of heparinised blood or synovial fluid and cultured in RPMI 1640 supplemented with 2 mM glutamine, 100 U ml^{-1} penicillin and streptomycin (Gibco BRL, Gaithersburg, MD USA) and 10% AB-positive heat-inactivated human serum (Sanquin Blood Bank, Utrecht, the Netherlands). Stimulation with peptides at 20 µg ml^{-1} was done in 96 wells round bottom plates for 96 hours at $2x10^5$ cells (200 µl per well) followed by analysis of peptide-specific proliferation of PBMC as described below. Additionally, the peptide-specific response of SFMC was measured by intracellular staining and FACS analysis. Therefore, after the first 96 hours of stimulation with peptides, 100 µl medium was replaced by fresh culture medium supplemented with 80 IU ml^{-1} IL-2, resulting in a final concentration in the well of 40 IU ml^{-1}. At day eight, medium was replaced by new medium along with fresh irradiated APC of the same donor ($1.5x10^5$ per well irradiated at 3500 rad). During 6 hours cells were cultured with or without the peptides and addition of the costimulatory mAbs CD28 and CD49 (0.5 µg ml^{-1} each). Moreover, Golgistop (BD Biosciences) was added during the last 4 hours. Harvested cells were surface stained with anti-CD3-Peridinin-chlorophyll-protein Complex (PerCP), anti-CD4-allophycocyanin (APC) and anti-CD69-fluorescein isothiocyanate (FITC) and subsequently fixed in Cytofix/cytoperm solution (BD Biosciences) followed by intracellular staining with phycoerythrin (PE) conjugated mAbs against either IL-10, IFN-γ or TNF-α. All antibodies were obtained from Becton Dickinson.

Antigen-specific T cell proliferation and cytokine production by murine cells

To determine antigen-specific proliferation, cultures were stimulated in triplicate with antigen or peptides (20 µg ml^{-1} unless stated otherwise). Single-cell suspensions of spleens or LN were cultured for 72 hours in 96-well flat bottom plates at $2x10^5$ cells per well in IMDM supplemented with 10% FBS (Bodinco B.V., Alkmaar, the Netherlands), 100 units ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, and $5x10^{-5}$ M 2-mercaptoethanol. Isolated human PBMC

were cultured for 96 hours. After 72 or 96 hours cells were pulsed overnight with [^{3}H]-thymidine (0.4-1 µCi per well; Amersham Biosciences Europe GmbH, Roosendaal, the Netherlands) and incorporation was measured. Data are expressed as mean stimulation index (mean cpm of cells cultured with antigen or peptide divided by mean cpm of cells cultured without peptide). To address antigen-specific IFN-γ, IL-10, IL-4 and IL-5 production, upon immunization or PGIA induction, supernatants of stimulated spleen cell cultures were collected for cytokine assays after 72 hours. Cytokine production was measured by ELISA according to manufactures protocol (BD).

Generation and testing of mC1b-specific hybridoma

Spleen cells from mice, immunized on day 0 and day 14 with 25 µg mC1b peptide mixed with 2 mg DDA emulsified in PBS, were isolated on day 28 and restimulated with 4 µg ml^{-1} mC1b in X-vivo-15 medium (Biohitaker) supplemented with glutamax, 100 units ml^{-1} penicillin and 100 µg ml^{-1} streptomycin for 48 hours. Then, viable cells were isolated using LympholyteM (Cedarlane Laboratories, Westbury, NY) and cultured for 48 hours in conditioned medium, IMDM supplemented with 10% FBS, 100 units ml^{-1} penicillin, 100 µg ml^{-1} streptomycin and 5 x 10^{-5} M 2-ME and 10% ConA-activated rat spleen supernatant as a source of T cell growth factors. Subsequently, spleen cells were fused with the fusion partner BW5147 as described previously (41). By FACS Vantage (BD) fused cells were seeded into 96 wells plates at one cell per well followed by analysis of mC1b specificity of the obtained clones. One of the mC1b-specific clones, designated LHEP7, was used in the experiments shown in this study. The 5/4E8 hybridoma (42) specific for PG_{70-84}, a proteoglycan-derived peptide, was used as control. Hybridoma cells (2x10^4 per well) were cultured with APC (A20 B lymphoma cells, 2x10^4 per well; obtained from the American Type Culture Collection) in 96 wells flat bottom plates for 24 hours with or without peptides at indicated concentrations. Additionally, A20 cells were heat shocked (one hour in a water bath at 42.5 ℃) followed by 2-3 hours recovery at 37℃ ºC and were afterwards used as APC. After 24 hours, harvested supernatants were cultured with IL-2 responder CTLL-16 cells (1x10^4 cells per well) for 24 hours, pulsed overnight with ^{3}H-thymidine followed by analysis of tritium incorporation (43).

Statistical analysis

Unless stated otherwise, data are expressed as mean ± standard error of the mean (s.e.m.). Statistical analysis was carried out using Prism software (version 4.00, Graphpad Software Inc., San Diego). Significance level was set at $p < 0.05$ and two tailed Students T test or one way Anova with Bonferroni correction were applied.

Results

Identification of C1 as a dominant and cross-reactive Mt Hsp70 T cell epitope

Previously, immunization with Mt Hsp70 has been shown to suppress proteoglycan- and adjuvant-induced arthritis (35, 44). In the present study we explored the dominant T cell epitopes of Mt Hsp70 by epitope mapping. *In vitro* we restimulated spleen and lymph node (LN) cells from Mt Hsp70 immunized mice with 123 15mer overlapping peptides covering the complete Mt Hsp70 protein and analyzed T cell proliferation. In several experiments 13 peptides repeatedly showed increased proliferation as compared to unstimulated conditions (Table 1). These peptides were selected as immunodominant peptides. As Hsp70 is highly conserved and cross-reactivity between bacterial- and self-Hsp is considered to be important for the regulatory potential of Hsp70 (29), these 13 peptides were divided in three pools based on homology between the bacterial and the mouse Hsp70 sequence; non-conserved peptides (group A), partly-conserved peptides (group B) and very-conserved peptides (group C).

Table 1. Identification of dominant Hsp70 T cell epitopes.

Peptide name	Sequence	Position	Proliferation	Identities*
A1	YTAPEISARILMKLK	86-100	1.6 (0.1)	n.d.
A2	KPFQSVIADTGISVS	291-305	2.8 (0.7)	n.d.
A3	AEGGSKVPEDTLNKV	530-544	2.7 (0.7)	n.d.
A4	AQAASQATGAAHPGG	585-599	2.5 (0.8)	n.d.
B1	EGSRTTPSIVAFARN	31-45	2.3 (0.7)	10/15
B2	MQRLREAAEKAKIEL	231-245	2.8 (0.3)	12/15
B3	GGKEPNKGVNPDEVV	331-345	2.0 (0.5)	10/15
B4	DEVVAVGAALQAGVL	342-356	2.3 (0.7)	11/15
B5	LDVTPLSLGIETKGG	366-380	1.5 (0.4)	14/15
C1	VLRIVNEPTAAALAY	141-155	4.0 (1.3)	13/15
C2	ILVFDLGGGTFDVSL	166-180	2.1 (0.4)	15/15
C3	RGIPQIEVTFDIDAN	441-455	2.0 (0.5)	14/15
C4	QIEVTFDIDANGIVH	445-459	1.6 (0.2)	15/15

To identify the dominant T cell epitopes of Mt Hsp70, mice were immunized on day 0 and day 14 with Mt Hsp70 in DDA. On day 28 spleen cells were isolated and restimulated with a panel of 123 overlapping 15mer peptides covering the complete sequence of Mt Hsp70. Subsequently induced T cell responses were detected by [^{3}H]-thymidine incorporation. The 13 peptides depicted in table 1 repeatedly induced proliferation upon *in vitro* restimulation with the peptides and were therefore selected as dominant epitopes. Data are expressed as mean stimulation index (cpm with antigen/cpm of medium only) (± s.e.m.) of n=3 mice and are representative of three experiments. * maximum amino acid identities with a mouse Hsp70 homologue. N.d., not detectable

To further characterize dominant T cell responses and their nature, we immunized mice with each of the three peptide pools and subsequently isolated cells from spleen, mesenteric or auxiliary LNs. Upon restimulation with peptides or antigens we measured cytokine (IFN-γ, IL-10, IL-4 and IL-5) production and proliferation. As expected, restimulation with the whole Mt Hsp70 protein increased the production of IFN-γ (Figure 1A) and IL-10 (Figure 1B) as compared to medium or Ova restimulation in all immunization groups. No obvious differences in these responses were found between the immunization groups. When restimulation with the 13 bacterial peptides was done individually, in each pool one of the peptides (peptide A2 in group A, B4 in group B and C1 in group C) dominantly induced proliferation of spleen or LN cells (data not shown) and production of IFN-γ by spleen cells (Figure 2A). Furthermore, both A2 and C1 peptide increased the production of IL-10 (Figure 2B), while this was not observed with the B4 peptide or any of the other peptides. For all peptides, restimulation with a peptide did not induce any response when mice were immunized with one of the peptide pools that did not include the restimulation peptide. None of the peptides augmented the expression of IL-4 or IL-5 (data not shown), indicating that none of the peptides induced a strong Th2 response. In addition to T cell responses induced by the bacterial-peptide, we studied cross-reactive responses to the mouse homologue peptides. Especially restimulation with the highly conserved mouse homologue peptides mC1a and mC1b (amino acid sequences in Table 2) induced production of IFN-γ (Figure 2C) and IL-10 (Figure 2D) in cultures obtained from spleens of mice immunized with the peptide pool containing the C1 peptide. This established that C1 primed T cells were also responsive to the mouse homologues. In summary, we found three dominant Mt Hsp70-derived T cell epitopes, with different peptide-induced cytokine profiles upon activation. Only the highly conserved C1 peptide induced cross-reactive T cell responses.

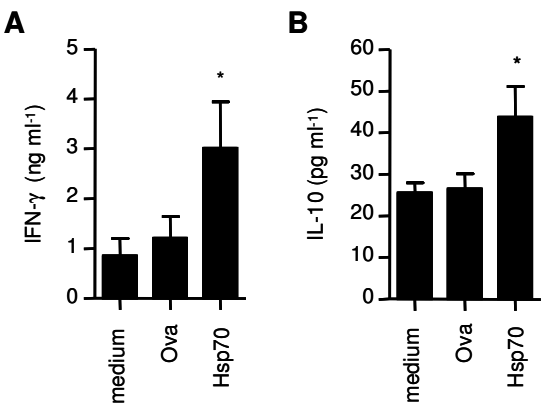

Figure 1. Characterization of peptide-specific T cell responses. Pools of immunodominant peptides (Table 1), containing 4-5 peptides were made, based on homology between mouse- and bacterial-Hsp70. Mice were immunized with one of the pools and subsequently isolated spleen cells were restimulated with the whole Mt Hsp70 protein or Ova as control protein. After 72 hours cytokine secretion was measured in the supernatant by ELISA. (A) IFN-γ and (B) IL-10. Data show mean cytokine production (± s.e.m.) of n=9 mice immunized with either peptide pool A, B or C. Statistical differences $p<0.05$ is indicated by *.

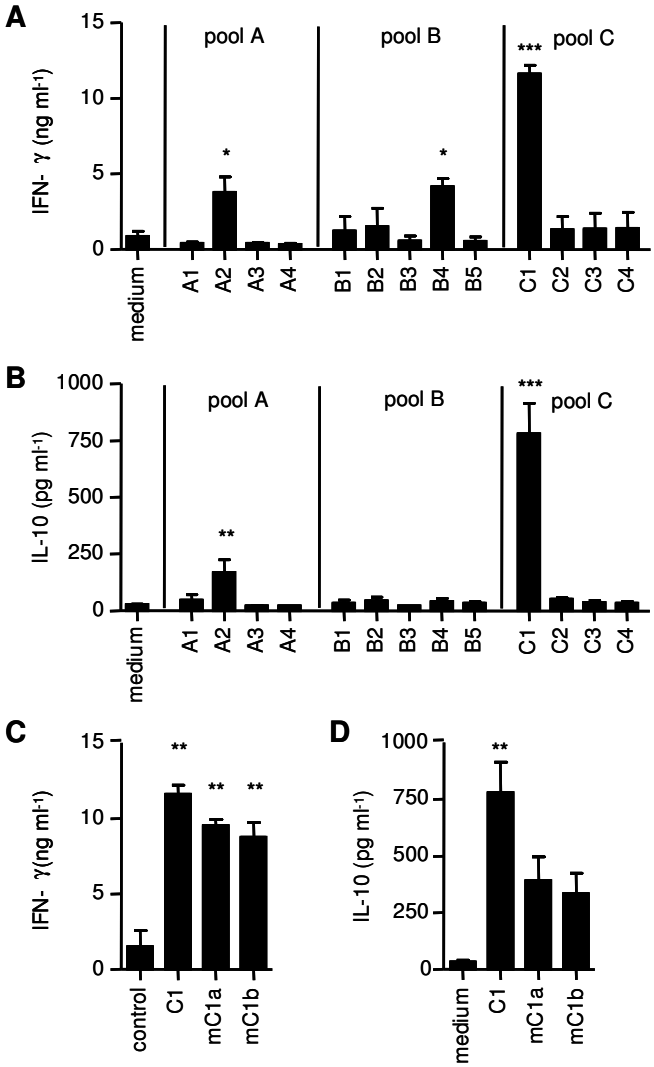

Figure 2. Characterization of dominant peptide-specific T cell responses. Pools containing 4-5 dominant peptides were made, based on homology between mouse and bacterial Hsp70. Mice were immunized with one of the pools. Isolated spleen cells were restimulated with Hsp70 peptides individually. After 72 hours cytokine secretion was measured in the supernatant by ELISA. (A) IFN-γ and (B) IL-10 production of spleen cells after restimulation with medium only or immunodominant bacterial-Hsp70 peptides. The depicted data only show the average cytokine production (± s.e.m.) after restimulation with peptides that were included in the immunization pool, n=3 mice per immunization group. (C) IFN-γ and (D) IL-10 production by spleen cells from mice immunized with pool C after restimulation with C1 or with the mouse homologue peptides mC1a and mC1b. Indicated is the mean cytokine production (± s.e.m.) of n=3 mice. Statistical differences are indicated by * $p<0.05$, ** $p<0.01$ and *** $p<0.001$

Table 2. Origin and sequence of highly conserved C1 peptides.

Peptide	Protein	Origin	ID	sequence
C1	DnaK (Hsp70)	*Mycobacterium tuberculosis*	885946	VLRIVNEPTAAALAY
mC1a	HspA9 (GRP75)	*Mus musculus*	15526	VLR**VI**NEPTAAALAY
		Homo sapiens	3313	
mC1b	HspA1A (Hsp72)	*Mus musculus*	193740	VLRI**I**NEPTAAA**I**AY
		Homo sapiens	3303	
	HspA8 (Hsc70)	*Mus musculus*	15481	VLRI**I**NEPTAAA**I**AY
		Homo sapiens	3312	

Origin and sequences of highly conserved C1 peptides used in the present study are depicted. Human and mouse peptides of the same protein were completely identical. Altered residues as compared to *Mycobacterium tuberculosis* in bold and underlined. * ID: GeneID in the NCBI Entrez Gene database (www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene)

Cross-reactive mC1b-specific T cells respond to up-regulation of endogenous Hsp70

For induction and activation of anti-inflammatory Hsp-specific T cell responses by bacterial-Hsp70, also the recognition of self-Hsp, over-expressed at the inflammatory site, is thought to be important (29). To acquire additional evidence that T cells specific for self Hsp70 peptides can recognize both the bacterial and the endogenous peptide, we generated a $CD4^+$ T cell hybridoma that recognized the mC1b peptide. This peptide was derived from the most abundant inducible mouse Hsp70 (HspA1A/B amino acids 169-183). First, mC1b peptide-specificity of the hybridoma was studied. Hybridoma activation was measured by increased secretion of bio-active IL-2 in the supernatant, as detected by augmented proliferation of the IL-2 responder line CTLL-16 (43). We found that hybridoma activation was increased after incubation with mC1b but not with PG_{70-84}, a proteoglycan (PG) peptide (aa 70 to 84) used as control peptide (Figure 3A). Furthermore mC1b recognition was completely blocked by incubation with M5/114, an antibody that blocks the two MHC class II molecules in BALB/c mice, $I\text{-}A^d$ and $I\text{-}E^d$. In contrast, hybridoma activation by ConA was not influenced by incubation with M5/114. Then, we addressed cross-recognition of the two homologues of mC1b and found that both the mouse homologue mC1a and the bacterial homologue C1 dose dependently enhanced the production of IL-2 (Figure 3B). This showed that indeed our self mC1b-specific hybridoma can cross-recognize the homologous peptides. Subsequently, we analyzed whether the C1 epitope was also presented in MHC class II upon up-regulation of endogenous Hsp70 as observed during inflammation. We exposed A20 lymphoma cells to heat stress (one hour heat shock at 42.5°C). Heat shock treatment of the APC enhanced the expression of Hsp70 (15% Hsp70 positive cells after heat shock versus 2.2% in control APC) in the APC. mC1b-specific hybridoma T cells produced enhanced levels of IL-2 in the presence of heat treated APC as compared to medium treated APC kept at 37°C (Figure 3C), whereas a control hybridoma specific for the

PG_{70-84} peptide did not respond to the mC1b or heat shocked APC (Figure 3D). Our data show that $CD4^+$ T cells with specificity for the mC1b peptide are cross-reactive with the bacterial C1 peptide and the mouse mC1a peptide and can actually recognize up-regulation of the endogenous Hp70 protein.

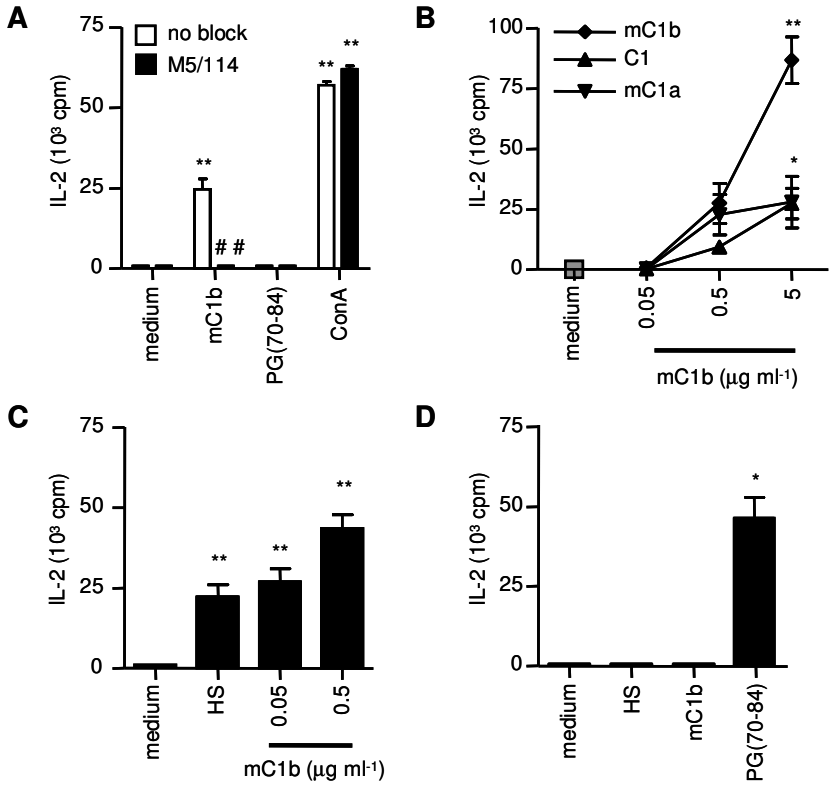

Figure 3. Up-regulation of endogenous Hsp70 activates cross-reactive mC1b-specific $CD4^+$ T cell hybridoma. (A-D) $CD4^+$ T cell hybridoma specific for (A-C) mC1b (aa 169-183) or (D) as a control, a PG peptide ($PG_{(70-84)}$) were cultured in the presence of irradiated APC (A20 cells). After overnight culture IL-2 production by activated hybridoma cells was determined as proliferation ($[^3H]$-incorporation) of the IL-2 dependent CTLL-16 line. All data are expressed as mean 10^3 cpm (± sd) of triplicate cultures and are representative for at least two experiments. (A) mC1b or as a control $PG_{(70-84)}$ were added at 0.5 µg ml^{-1}. ConA (2 µg ml^{-1}) was used as positive control. To block MHC class II peptide presentation, to some cultures M5/114 antibody was added. (B) Cross-reactive responses to mC1b homologue peptides were measured by incubation with the peptides at indicated concentrations. (C-D) A20 cells were exposed to one hour heat shock at 42.5°C. Control APC were kept at 37°C. After three hours recovery at 37°C, A20 cells were cultured with (C) mC1b-specific hybridoma or (D) as a control with a hybridoma recognizing $PG_{(70-84)}$. Significant differences are indicated by * $p<0.05$ or ** $p<0.01$ compared to medium control 37°C APC and in figure A ## $p<0.01$ compared to mC1b incubation without M5/114.

(m)C1 peptide-specific responses in human PBMC and SFMC

In order to act as potential candidate peptides, relevant for epitope-specific immune therapy in humans, the peptides must be recognized by the human immune system. Since the C1 region of Mt Hsp70 is highly conserved, the mouse homologue peptides mC1a and mC1b are completely identical to the human peptide. To address if these peptides could actually bind to the human MHC class II we determined theoretical HLA-DR binding scores with a motif based prediction algorithm (SYFPEITY) database. C1, mC1a and mC1b were found to bind *in silico* to several HLA-DR molecules (Table 3), confirming the observation that mC1 like peptides have been eluted from several HLA-DR molecules [supplementary table 1].

Table 3. *In silico* bindings scores.

peptide	Protein	Origin	DRB1 *0101	DRB1 *0301	DRB1 *0401	DRB1 *0701	DRB1 *1101	DRB1 *1501
C1	HspA1A	*Mycobacterium tuberculosis*	23	14	26	20	12	18
mC1a	HspA9	*Mus musculus* *Homo sapiens*	22	14	26	20	12	18
mC1b	HspA1A	*Mus musculus* *Homo sapiens*	22	14	26	20	12	18

Theoretical HLA-DR binding scores were determined with a motif based prediction algorithm (SYFPEITHI) database (http://www.syfpeithi.de). *In silico* bindingscores for DRB1*0101, *0301, *0401, *0701, *1101 and *1501 are depicted for C1, (*Mycobacterium tuberculosis* DnaK), mC1a (*Mus musculus* HspA9) and mC1b (*Mus musculus* HspA1A). Amino acid sequences of *Mus musculus* and *Homo sapiens* mC1a/b peptides are completely identical.

To obtain evidence that (m)C1 peptides can indeed be recognized by human T cells, peripheral blood mononuclear cells (PBMC) obtained from healthy controls were stimulated with the peptides. Incubation with mC1b but not mC1a, increased proliferation compared to the A5 control peptide, an adenovirus-derived peptide (Figure 4A). In an earlier study $CD4^+$ T cells recognizing Hsp60 have been shown in the inflamed joint (24). Upon activation, these T cells may locally suppress inflammation. Therefore, we tested if T cells were present in the synovium that could recognize (m)C1 peptides. For this, we isolated synovial fluid mononuclear cells (SFMC) of a JIA patient and stimulated the cells with the peptides. Stimulation with the mC1b peptide induced the production of IL-10, IFN-γ and TNF-α and surface expression of the T cell activation marker CD69 in the $CD3^+CD4^+$ population (Figure 4B-C). Also, the bacterial C1 peptide enhanced CD69 and cytokine expression. In contrast, the control peptide A5 and mC1a did not induce a peptide-specific response. Similar results were obtained when SFMC from a RA patient were used (data not shown). In summary this shows that the C1 and especially mC1b are recognized by human T cells both in healthy controls and in the inflamed joint, illustrating the relevance of the peptides for the human immune system.

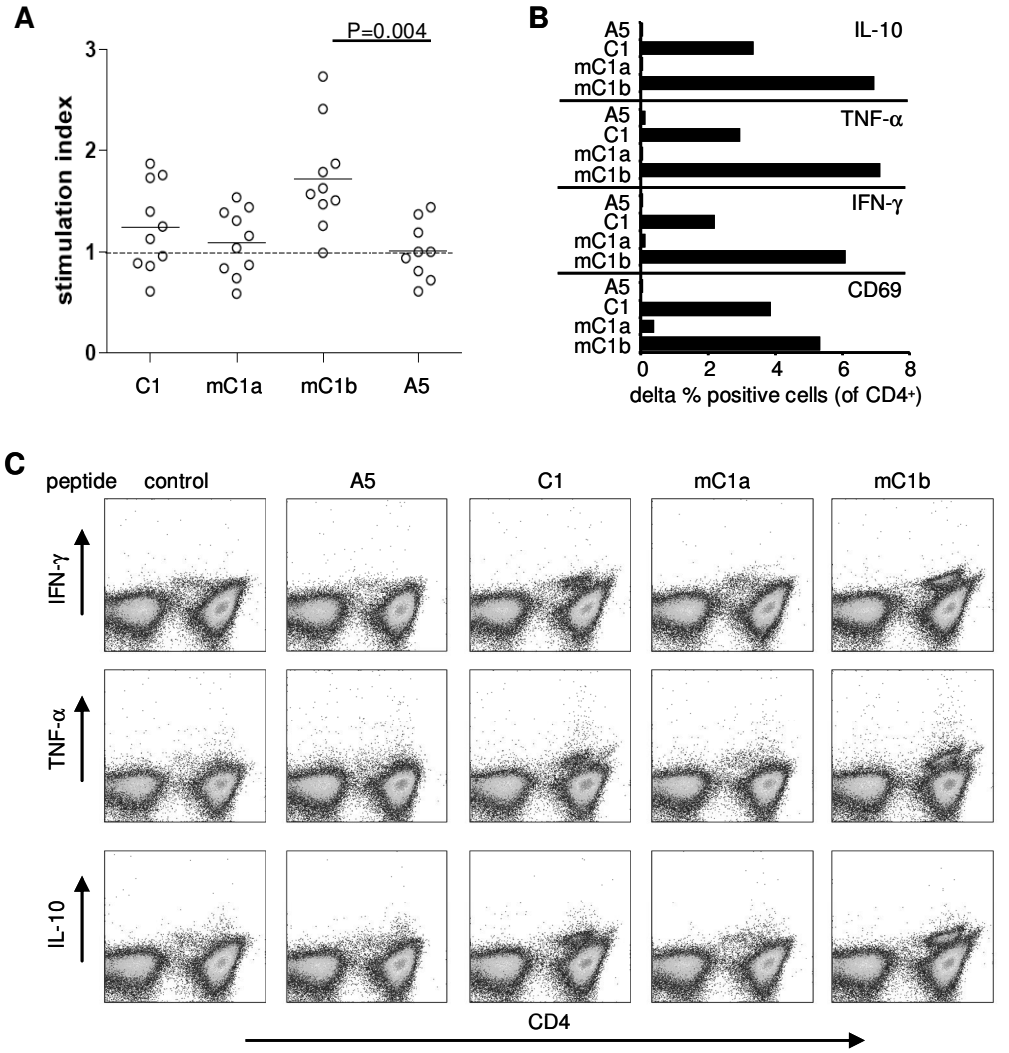

Figure 4. (m)C1 peptide-specific responses in human PBMC and SFMC. To explore peptide-specific responses in humans (A) proliferation of PBMC isolated from the blood of healthy donors was determined by measuring [^{3}H]-incorporation after 96 hours incubation with C1, mC1a, mC1b or as a control A5 peptides (20 µg ml^{-1}). Results are expressed as mean stimulation index (cpm Antigen/cpm background). Statistical differences were calculated by Wilcoxon signed rank test. (B-C) In SFMC obtained from a JIA patient, peptide-induced intracellular cytokine production and expression of the early T cell activation marker CD69 were studied by flow cytometry after eight days culturing in the presence of peptides as described in materials and methods. During the last 6 hours fresh medium, APC and the co-stimulatory mAbs CD28 and CD49 were added (control cultures), and in peptide induced cultures also C1, mC1a, mC1b or A5 (20 µg ml^{-1}) were added. Data in B are expressed as delta % positive cells from CD3$^+$CD4$^+$ population (% positive of peptide - % positive of control). Plots in C show cytokine production in the CD3$^+$ population.

Suppression of arthritis by administration of Mt Hsp70 or Hsp70-derived peptides

To explore the immunoregulatory capacity of (m)C1 peptides in inflammatory disease we used the PGIA model that shares many characteristics with human RA (45, 46). In a previous study in this model we found that immunization with whole Mt Hsp70 suppressed arthritis (35). Since especially the mucosal administration route primes toward the induction of Treg and allows easy administration of the peptide (47) we first tested whether Mt Hsp70 was also effective through intranasal (i.n.) administration. Thus, Mt Hsp70 or PBS, as a control, were i.n. given to BALB/c mice on day -7, -5 and -3. Subsequently, on day 0 and day 21 arthritis was induced. Nasal application of Mt Hsp70 delayed the onset of disease (Figure 5A), decreased the maximum arthritis score (Figure 5B) and suppressed overall arthritis severity as scored by clinical examination of the paws (Figure 5C).

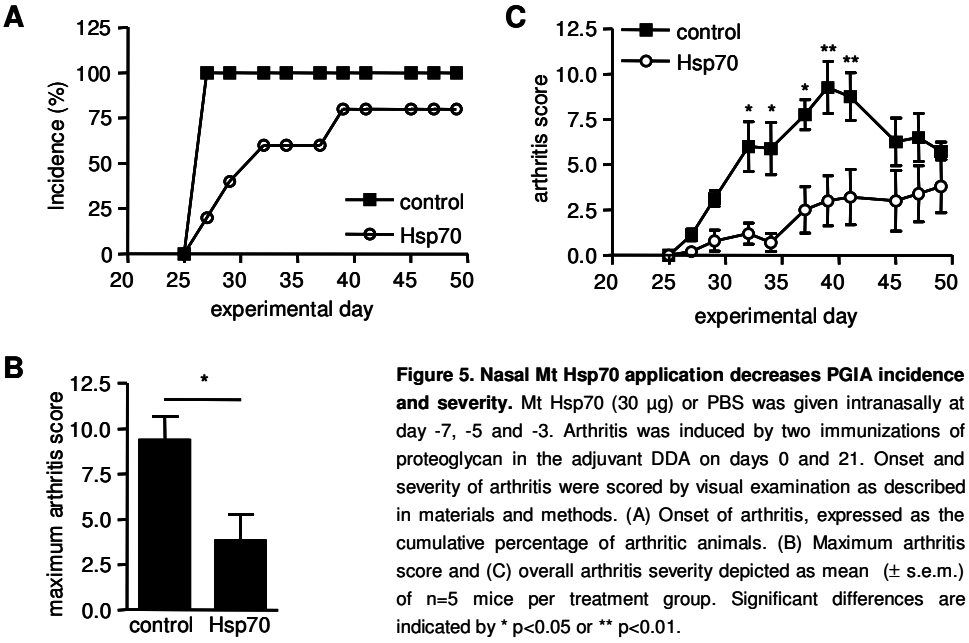

Figure 5. Nasal Mt Hsp70 application decreases PGIA incidence and severity. Mt Hsp70 (30 µg) or PBS was given intranasally at day -7, -5 and -3. Arthritis was induced by two immunizations of proteoglycan in the adjuvant DDA on days 0 and 21. Onset and severity of arthritis were scored by visual examination as described in materials and methods. (A) Onset of arthritis, expressed as the cumulative percentage of arthritic animals. (B) Maximum arthritis score and (C) overall arthritis severity depicted as mean (± s.e.m.) of n=5 mice per treatment group. Significant differences are indicated by * $p<0.05$ or ** $p<0.01$.

Next, we studied the regulatory potential of the Hsp70 peptides. Therefore, we treated mice i.n. with C1, mC1a or mC1b peptide or with $Ova_{323-339}$, as control peptide and induced PGIA as described in figure 5. Nasal application of C1 peptide suppressed clinical symptoms of arthritis as compared to $Ova_{323-339}$ treated mice (Figure 6). Also, pretreatment with mC1a decreased arthritis severity. Although the reduction of overall arthritis severity by mC1b peptide was not significant, it delayed the onset of arthritis: day 31.1 (± 1.8) compared to day 26.5 (± 0.5) in mice that had received $Ova_{323-339}$ ($p<0.05$) (Table 4).

Table 4. Nasal peptide application suppresses PGIA.

Treatment group	Onset	Max. arthritis score
$Ova_{(323-339)}$	26.5 (0.5)	8.0 (0.7)
C1	31.5 (1.4) *	4.0 (0.9) *
mC1a	28.2 (1.8)	4.0 (1.4) *
mC1b	31.1 (1.8) *	6.8 (1.7)

C1, mC1a, mC1b or control ($Ova_{323-339}$) peptide (100 µg) were given intranasally on day -7, -5 and -3 and arthritis was induced by PG/DDA immunization on day 0 and day 21. Arthritis symptoms were scored as described in materials and methods. Day of onset and maximum arthritis score were depicted as means (± s.e.m.) of n=6 mice. * $p<0.05$, as compared to $Ova_{323-339}$ group.

In addition, we studied induced peptide-specific T cell differentiation by analysis of peptide-specific proliferation and cytokine production by spleen cells isolated on day 28 after the final PG immunization. *In vitro* restimulation with C1, and with mC1a and mC1b, clearly amplified proliferation of spleen cells from mice that had received C1 peptide compared to mice that had received $Ova_{323-339}$ (Figure 7A). This was also detected by analysis of IFN-γ (Figure 7B) and IL-10 (Figure 7C) production upon C1 restimulation and showed that i.n. C1 administration had induced a strong cross-reactive T cell response. Remarkably, even though the mC1a peptide suppressed PGIA, no peptide-specific T cell responses were observed in this group. Although, T cell responses induced by mC1b peptide application, were less prominent then C1 induced responses, restimulation with mC1b enhanced proliferation, IFN-γ and IL-10 production as compared to the control $Ova_{323-339}$ group. Additionally, the cross-reactive response towards C1 and mC1a tended to be increased. Combined these data demonstrated the immunomodulatory capacity of the (m)C1 peptides.

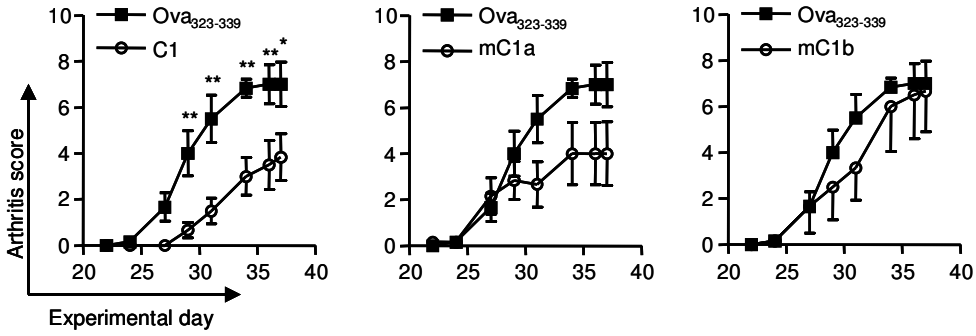

Figure 6. Nasal peptide application suppresses PGIA. C1, mC1a, mC1b or control ($Ova_{323-339}$) peptide (100 µg) were given intranasally on day -7, -5 and -3 and arthritis was induced by PG/DDA immunization on day 0 and day 21. Arthritis symptoms were scored as described in materials and methods and expressed as mean arthritis score (± s.e.m.). Data are representative of two independent experiments (n=6 mice per group) Significant differences compared to $Ova_{323-339}$ control group are indicated by * $p<0.05$ or ** $p<0.01$.

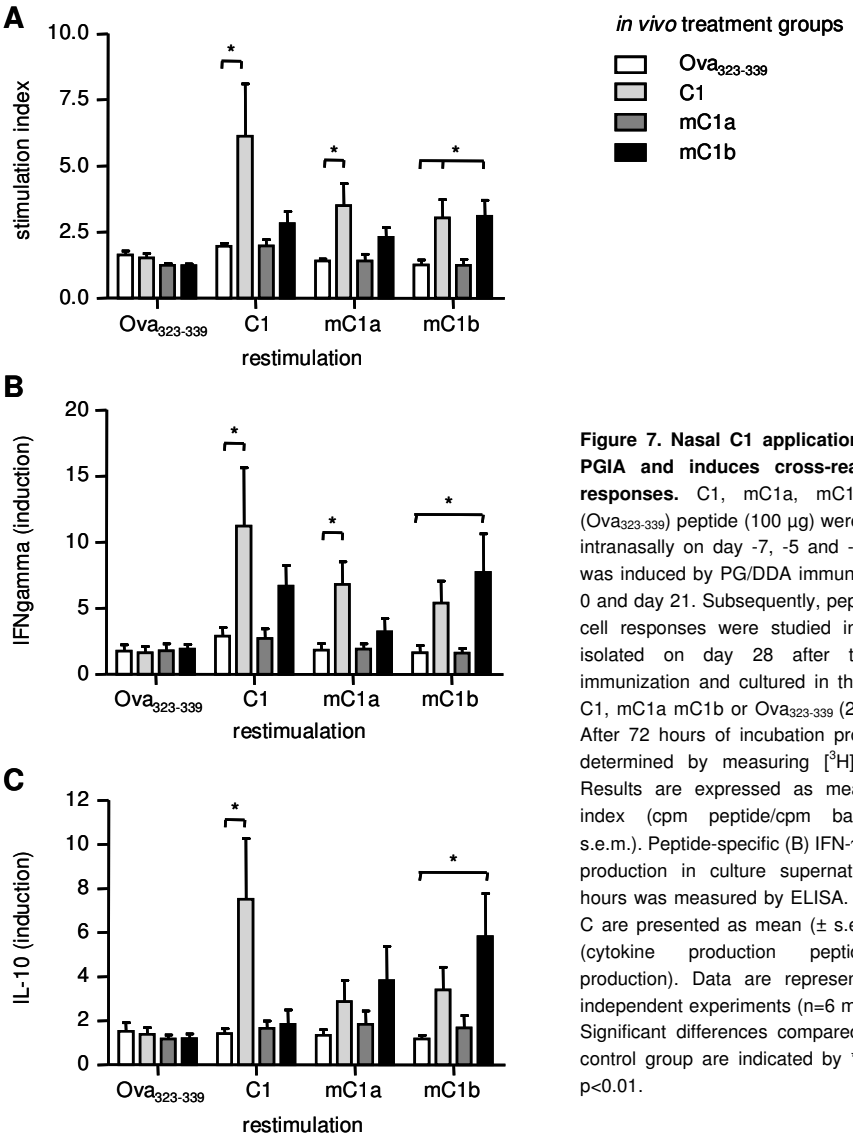

Figure 7. Nasal C1 application suppresses PGIA and induces cross-reactive T cell responses. C1, mC1a, mC1b or control ($Ova_{323-339}$) peptide (100 µg) were administered intranasally on day -7, -5 and -3 and arthritis was induced by PG/DDA immunization on day 0 and day 21. Subsequently, peptide-specific T cell responses were studied in spleen cells isolated on day 28 after the final PG immunization and cultured in the presence of C1, mC1a mC1b or $Ova_{323-339}$ (20 µg ml^{-1}). (A) After 72 hours of incubation proliferation was determined by measuring [^{3}H]-incorporation. Results are expressed as mean stimulation index (cpm peptide/cpm background) (± s.e.m.). Peptide-specific (B) IFN-γ and (C) IL-10 production in culture supernatants after 72 hours was measured by ELISA. Data in B and C are presented as mean (± s.e.m.) induction (cytokine production peptide/background production). Data are representative of two independent experiments (n=6 mice per group) Significant differences compared to $Ova_{323-339}$ control group are indicated by * $p<0.05$ or ** $p<0.01$.

Discussion

In the present study we investigated an Hsp70-derived peptide that fulfills several characteristics of antigens that can be used for epitope-specific therapy in chronic inflammatory disease. The C1 peptide is located in a highly conserved part of the inducible Mt Hsp70, enabling cross-reactive T cell responses between T cells specific for self and for bacterial-Hsp. Next, mC1b-specific T cells responded to increased cellular expression of Hsp70 indicating that the amount of peptide presented on MHC class II increased upon up-regulation of the endogenous Hsp70 protein. Furthermore, the immunomodulatory potential of the peptide was emphasized by suppression of PGIA upon i.n. administration of the peptide. Our observation that also in humans (m)C1 peptide-specific responses can be found shows the relevance of peptides from the C1 region for the human immune system.

Recently we showed that, in the mouse model of PGIA, the protective potential of Mt Hsp70 by i.p. immunization was IL-10 dependent (35). We now identified multiple dominant T cell epitopes of Mt Hsp70 in BALB/c mice. However, only one of the highly conserved epitopes, peptide C1, induced high levels of IL-10. This suggested a potential immunoregulatory function of this peptide. Indeed, i.n. application of C1 decreased PGIA severity. Peptide-specific suppression of arthritis by conserved peptides has also been shown in adjuvant induced arthritis in Lewis rats after immunization with an Hsp70-derived peptide (234-252) (34) or after nasal treatment with a conserved Hsp70 peptide (p111) (28). Like C1, p111 and its rat homologue peptide R111 induced the production of IL-10 by p111-specific T cells. This peptide was also located in the conserved N terminal region of the protein; however, in the present study we did not identify this peptide as a dominant epitope in BALB/c mice. In the PGIA model we also found that cross-reactive T cell responses towards C1, mC1a and mC1b were induced by i.n. administration of the bacterial C1 peptide. Although less pronounced, the reverse was also observed for mC1b. Strikingly, no peptide-specific responses were detectable upon pretreatment with the mC1a peptide, even though it suppressed PGIA. Although we can not fully explain the observed differences between peptide induced T cell responses, the two amino acid differences between mC1a and mC1b or C1 could influence induced T cell responses leading to different T cell activation profiles upon *in vitro* restimulation.

Cross-reactivity is thought to be important for induction and boosting of Hsp-specific immunoregulation (29, 48, 49). Bacterial Hsp-specific T cells will be induced upon encounter of bacterial-Hsp during infection and at mucosal surfaces such as the gut. In the gut, through mechanisms of mucosal tolerance, these T cells will be directed towards a regulatory phenotype. In addition, in response to stress self-Hsp will be upregulated and T cells specific for conserved bacterial sequences can recognize self Hsp that can function as an altered peptide ligand (29). Cross-reactivity was demonstrated in the present study by the

observation that C1-specific T cells, induced by immunization with the C1 peptide, produced high levels of IFN-γ and IL-10 after *in vitro* restimulation with C1 and its mouse homologue peptides mC1a and mC1b. None of the other highly conserved peptides clearly induced IFN-γ or IL-10 themselves or upon restimulation with cross-reactive peptides. The B4 peptide enhanced IFN-γ but not IL-10 production. But, this increase in IFN-γ was almost absent in response to B4 homologue peptides (data not shown). We additionally found cross-reactive responses with a mC1b-specific $CD4^+$ T cell hybridoma that was also activated by C1 (bacterial derived) and by mC1a (mouse HspA9). In our study we only addressed these two mouse homologues but, members of the Hsp70 family in mice are encoded by at least 13 Hsp70 genes leading to multiple inducible and non-inducible Hsp70 proteins (9, 50). In addition to being conserved among species, the C1 region is also highly conserved between the different Hsp70 genes. Therefore, it will be interesting to study if also peptides from other Hsp70 family members, such as BiP (HspA5), are cross-recognized.

Enhanced expression of several Hsp family members has been reported under inflammatory conditions. Increased Hsp60, Hsp70 and BiP expression has been observed in the inflamed synovium of JIA and RA patients (18-20, 51) and will be important for Hsp-specific T-cells to exert their regulatory function. In order to activate $CD4^+$ T cells, peptides from the up-regulated Hsp70 must be processed and presented on MHC class II. We previously showed that Hsp60-specific T cells recognized enhanced Hsp60 expression in heat stressed APC (52). In line with this, in the present study up-regulation of endogenous Hsp70 by the APC augmented mC1b-specific $CD4^+$ T cell activation in the present study. Our findings demonstrate that MHC class II presentation of the peptide is increased upon up-regulation of the protein and that T cells can actually respond to this increase. Usually, intracellular proteins, like Hsp70, are loaded on MHC class I. However, a literature study on Hsp-derived peptides eluted from MHC class II revealed that especially peptides originating from the C1 region have been frequently eluted from human MHC class II molecules (Supplementary table 1). How then do intracellular proteins end up in MHC class II? This might occur via a process named autophagy (53, 54). By macroautophagy cytosolic proteins are incorporated in autophagosomes that subsequently fuse with lysosomes. Alternatively, through chaperone mediated autophagy cytosolic proteins can be transported via LAMP2a directly from the cytosol into lysosomes followed by loading on MHC class II (55, 56). In a recent study in human HLA-$DR4^+$ B cells, increased amounts of especially Hsp70 peptides, including the mC1b peptide, were found in HLA-DR4 upon induction of autophagy by nutrient starvation (22). Because in that study nutrient starvation also increased cellular Hsp70 mRNA levels, it illustrates that enhanced cellular Hsp70 expression coincides with enhanced entering of the MHC classII loading pathway by the mC1b peptide. Thus, through autophagy, up-regulation of endogenous Hsp70 at the inflammatory site may lead to enhanced presentation of the mC1b peptide followed by increased recognition of mC1b-specific T cells.

To address if (m)C1 peptides in the context of HLA could be targets for human T cells, we first measured *in silico* HLA binding using a computer algoritm, and found that peptides from the C1 region were panDR binders. Given the highly polymorphic nature of the human HLA, panDR binding peptides will increase the likelihood of successful therapy in multiple individuals with differential HLA expression. Peptide stimulation of PBMC from healthy controls with C1 and mC1b peptide showed that indeed human T cells could recognize the peptides. Previously in another study, in healthy Caucasian BCG vaccines $N15_{(141-160)}$ was found as one of the dominant T cell epitopes of *Mycobacterium leprae* Hsp70, in the context of multiple HLA- DR types (57). This peptide comprises C1 identified in the present study as the dominant epitope from *Mycobacterium tuberculosis* Hsp70 which underlined the panDR binding capacity of the peptide.

Hsp-derived peptide-specific T cell responses have been described in chronic inflammatory diseases. For example, BiP-specific T cell responses have been observed in RA patients (51) and Hsp60-directed T cell responses have been associated with a benign and remitting from of JIA (9, 25, 58). In juvenile dermatomyositis (JDM), upon stimulation with several pan-DR binding Hsp60 peptides, the most distinct T cell responses were induced by the same peptides as in JIA (20). Additionally, Hsp70-specific T cell responses have been reported to be increased in children with Type 1 diabetes, as compared to healthy controls. In that study responses against peptide p12 (HspA1A / HspA8 residues 166-185) thus including the mC1b region, have been detected (59). Studies on associations between Hsp70 peptide-specific T cell responses and disease outcome, such as the ones described for Hsp60 peptides, have not been done yet. In our study, mC1b- and C1-specific T cell responses were also found in SFMC derived from the joint of a JIA and a RA patient by increased cytokine production upon peptide restimulation. This showed that T cells present at the site of inflammation can actually respond to the peptide but does not confirm any regulatory properties of these T cells. Further research is required to study this and associations between mC1b- or C1-specific responses and disease outcome.

Inflammatory stress enhanced Hsp expression and subsequently increased presentation of Hsp-derived peptides are postulated to trigger a naturally occurring feedback mechanism by the activation of Hsp-specific regulatory T cells (6, 60). We hypothesized that targeting this feedback system, by peptide boosting of (self) Hsp70-specific T cell immunity, would be an attractive therapeutic strategy in chronic inflammatory disorders. Besides peptide treatment alone, combination of peptide-specific therapy with established immunosuppressive agents might be a promising strategy. This has been demonstrated in rat adjuvant arthritis by the synergistic effects of combination treatment with an Hsp60 peptide and TNF-α inhibition (61). Such combination therapy will increase antigen-specific Treg responses and simultaneously allows lower dose of the immunosuppressive agent. Similar studies can be carried out with Hsp70 peptides.

In the present study we explored presentation of Hsp70-derived peptides and characterized peptide-specific T cell responses. Our findings that (m)C1 peptides were presented and recognized under physiological and inflammatory conditions together with their immunomodulatory capacity makes (m)C1 peptides interesting candidates for epitope-specific immunotherapy in chronic inflammatory diseases.

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Supplementary Table 1 Hsp70 peptides in MHC

Sequence	Class II type	Species	Source (100% id)	Entrez gene ID	Ref.
IIANDQGNRTTPSY	I-Ak	mouse	hspa8 (28-41)	15481	1
			hspa2 (29-42)	15512	
			hspa1l (30-43)	15482	
			hspa1a (28-41)	193740	
			hspa1b (28-41)	15511	
ITPSYVAFTPEGERL	I-Ab	mouse	hspa5 (62-76)	14828	2
TPSYVAFTDTERLIG	DRB1*0701	human	hspa8 (38-52)	3312	3
			hspa2 (39-53)	3306	
			hspa1l (40-54)	3305	
			hspa1a (38-52)	3303	
TPSYVAFTDTERLIGDA	DRB1*0701	human	hspa8 (38-52)	3312	3
			hspa2 (39-53)	3306	
			hspa1l (40-54)	3305	
			hspa1a (38-52)	3303	
DAAKNQLTSNPEN	I-Ag7	mouse	hspa5 (79-91)	14828	4
NPTNTVFDAKRLIGRRFD	HLA-DRB1*1104	human	hspa8 (62-79)	3312	5
QDIKFLPFKVVEKKTKPY	BoLA-DRB3*1201	bovine	hspa5 (111-128)	14828	6
LNVLRIINEPTAAAIAYG	HLA-DRB*0401	human	hspa8 (167-184)	24468	7
(NVLRIINEPTAAAIAYG)	(in rat line)		hspa1a (167-184)	24472	
			hspa1l (169-186)	24963	
			hspa2 (168-185)	60460	
NVLRIINEPTAAAIAYG	HLA-DRB1*0401	human	hspa8 (168-184)	3312	8
	HLA-DRB4*0101		hspa1a (168-184)	3303	
			hspa1l (170-186)	3305	
			hspa2 (169-185)	3306	
			hspa6 (170-186)	3310	
NVLRIINEPTAAAIA	multiple HLA mix	human	hspa8 (168-184)	3312	9
			hspa1a (168-184)	3303	
			hspa1l (170-186)	3305	
			hspa2 (169-185)	3306	
			hspa6 (170-186)	3310	
NVMRIINEPTAAAIAYG	multiple HLA mix	human	hspa5 (194-210)	3309	9
VMRIINEPTAAAIAYG	HLA-DRB1*0401	human	hspa5 (195-210)	3309	8
IINEPTAAAIAYGLD	HLA-DQ6 (B*602)	human	hspa8 (172-186)	3312	10
			hspa2 (173-187)	3306	
			hspa1l (174-188)	3305	
			hspa1a (172-186)	3303	
			hspa5 (198-212)	3309	

hspa1a=hsp70.1; hspa2=hsp70.2; hspa5=Bip; hspa8=hsc70;

Sequence	Class II type	Species	Source (100% id)	Entrez gene ID	Ref.
NRMVNHFIAEFKRK	I-Ek	mouse	hspa8 (236-249)	15481	11
RMVNHFIAEFKRKH	I-Ek	mouse	hspa8 (236-249)	15481	12
VNHFIAEFKRKHKKD	HLA-DR11/w52	human	hspa8 (238-252)	3312	13
XDFYTSITRAXFEE	HLA-DR11/w52	human	hspa8 (291-304)	3312	13
			hspa1a (291-304)	3303	
			hspa1l (293-306)	3305	
			hspa2 (294-307)	3306	
			hspa6 (294-306)	3310	
EGEDFSETLTRAKFEEL	BoLA-DRB3*1201 (in mus line)	bovine	hspa5 (315-331)	14828	6
ADLFRGTLDPVEK	HLA-DQ6 (B*0604)	human	hspa8 (307-319)	3312	10
TIPTKQTQTFTTYSDNQP	RT1.Bl	rat	hspa8 (419-436)	24468	14
			hspa1a (419-436)	24472	
VPTKKSQIFSTASDNQPTVT	HLA-DRB1*0401 DRB4*0101	human	hspa5 (443-462)	3309	8
GERAMTKDNNLLG	HLA-DR4Dw4 DRB1*0401	human	hspa8 (445-457)	3312	15
			hspa1a (445-457)	3303	
			hspa1l (447-459)	3305	
			hspa2 (448-460)	3306	
			hspa6 (447-459)	3310	
GERAMTKDNNLLGKFE	HLA-DRB1*0401 HLA-DRB4*0101	human	hspa8 (445-460)	3312	8
			hspa1a (445-460)	3303	
GERAMTKDNNLLGRFE	HLA-DRB1*0401 HLA-DRB4*0101	human	hspa6 (447-462)	3310	8
ANGILNVSAVDKSTGKE	HLA-DRB*0401	human	hspa8 (482-499)	3312	16
GILNVSAVDKSTGK	HLA-DRB*0401	human	hspa8 (484-497)	3312	16
GILNVSAVDKSTGKE	HLA-DRB1*0401 HLA-DRB4*0101	human	hspa8 (484-498)	3312	8
CNEIINWLDKNQ	HLA-DR4Dw10 DRB1*0402	human	hspa8 (574-585)	3312	15
ISWLDKNQTAEKEEFE	HLA-DQ8 (transgen in NOD!)	human	hspa8 (578-593)	15481	4
YGSGGPPPTGEEDTSEKDEL	I-Ag7	mouse	hspa5 (636-655)	14828	4
KELKIDIIPNPQER	HLA-DR4Dw15	human	hsp90ab1 (69-82)	3326	15
			hsp90bc (69-82)	3327	
GDEDASRMEEVD	I-Ep	mouse	hsp90ab1 (713-724)	15516	17
LYKEIYQTYGFLPS	I-Ep	mouse	hsp60 (27-40)	15510	17
TPEEIAQVATISANGDKD	I-Ag7	mouse	hsp60 (164-181)	15510	4
VNMVEKGIIDPTKVVRTALLD	I-Ep	mouse	hsp60 (511-531)	15510	17

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CHAPTER 7

The oregano constituent carvacrol boosts stress protein Hsp70 to activate T cell regulation of inflammation in autoimmune arthritis

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Abstract

Stress proteins, such as members of the heat shock protein (Hsp) families, are up-regulated by cells in the inflamed tissues and can be viewed functionally as 'biomarkers' for the immune system to monitor inflammation. Decreased stress induced expression of Hsp has been observed in immune disorders and in aged individuals. Here we hypothesized that a diminished stress response contributes to inflammatory disease susceptibility and that boosting of endogenous Hsp expression can restore effective immune regulation through T cells specific for stress proteins.

In the present study we manipulated stress protein expression *in vivo* with a food component, and studied immune recognition of stress proteins expressed in the tissue. We report that carvacrol, a major compound in oil of many *Origanum* species, had an unprecedented capacity to co-induce cellular heat shock protein 70 (Hsp70) expression *in vitro* and, upon intragastric administration, in Peyer's patches of mice *in vivo*. As a consequence, carvacrol specifically promoted T cell recognition of endogenous Hsp70 as shown *in vitro* by the activation of a Hsp70-specific T cell hybridoma and amplified T cell responses to Hsp70 *in vivo*. Carvacrol administration also increased the number of $CD4^+CD25^+Foxp3^+$ T cells, systemically in the spleen and locally in the joint, and almost completely suppressed proteoglycan-induced experimental arthritis. Furthermore, protection against arthritis could be transferred with T cells isolated from carvacrol-fed mice.

These findings illustrate that a food component can boost protective T cell responses to a self-stress protein and down-regulate inflammatory disease; the immune system responds to one's diet. Given the fact that carvacrol is a normal dietary constituent, the development of carvacrol as a novel preventive or therapeutic entity in rheumatoid arthritis and possibly other chronic inflammatory diseases seems realistic, attractive and relatively safe.

Introduction

Unhealthy diet, along with pro-oxidant life style, causes obesity and low-grade chronic inflammation associated with an increase in autoimmune diseases, atherosclerosis and allergies (1-3). Regulatory T cells (Tregs) that control inflammation (4) appears to offer new ways for preventive and therapeutic measures; activation of such Tregs by an appropriate diet could be attractive (5). In order to select the appropriate type of regulation the immune system must sense the location, kinetics and intensity of the inflammatory process (6). Stress proteins, such as members of the heat shock protein (Hsp) families, are up-regulated by cells in the inflamed tissues (7-9) and may constitute a reliable sensor system for the inflammatory state (10).

Both the innate and the adaptive immune system seems attuned to stress proteins and the outcome can be both immune stimulatory and inhibitory (11, 12). Stimulatory effects of Hsp have been exploited for example to enhance immunogenicity of tumour cells (13, 14). On the other hand, exogenous administration of stress proteins has produced immune regulation in various inflammatory models summarized in (15). For example vaccination with Hsp60 or an Hsp60 derived peptide (p227) rescued NOD mice from spontaneous development of type 1 diabetes (16) and C57BL/KsJ mice from streptozotocin induced diabetes (17). Also, immunization with gp96 suppressed NOD diabetes and murine encephalomyelitis (18). In several arthritis models, including adjuvant- (19, 20) and avridine-induced arthritis (21) in rats and collagen- (22) and proteoglycan-induced arthritis (23) in mice, exogenous Hsp administration suppressed arthritis. Furthermore, mucosal administration of Hsp60 has been shown to decrease inflammation and atherosclerosis in low-density lipoprotein receptor deficient mice (24, 25).

In contrast to experimental models, in humans the actual disease trigger is frequently unknown and variable which complicates antigen-specific therapy. The fact that stress proteins have been shown to suppress inflammation in such a variety of models suggests that Hsp-specific T cells act independently of the actual disease-inducing trigger. The potential of stress proteins for antigen-specific Treg therapy has been emphasized in initial clinical trials in humans with Hsp (26, 27). To enable local activation of antigen-specific Treg, candidate antigens for successful therapeutic intervention should be abundantly expressed at the site of inflammation (28). Therefore, overexpression of Hsp in inflamed tissues may be an important feature of Hsp mediated immuno modulation. Decreased stress-induced expression of Hsp has been observed in immune disorders (29, 30) and in aged individuals (31, 32). We therefore hypothesize that a diminished stress response contributes to inflammatory disease susceptibility and that the boosting of endogenous Hsp expression can restore effective immune regulation through T cells specific for stress proteins.

In the present study we manipulated stress protein expression levels with the food component carvacrol, a major compound in the essential oil of many *Origanum s*pecies (33). Essential oils are obtained by distillation of (dried) plant matter. Thus far, carvacrol mainly has been studied for its anti-bacterial effects (34). In a previous study in prokaryotic cells, carvacrol has been shown to enhance Hsp60 but not Hsp70 expression (35). We now have obtained evidence that carvacrol is a co-inducer of stress proteins in eukaryotes. In contrast to an Hsp inducer, which up-regulates Hsp expression by itself, a co-inducer will only increase Hsp levels in combination with a bona fide stress signal (36). In testing a larger set of potential food-derived Hsp (co-)inducers (data not shown) *in vitro*, we found that carvacrol is a remarkably potent enhancer of stress induced Hsp70 expression but does not induce Hsp70 expression by itself. We also studied the immune recognition of stress proteins expressed by the tissues *in situ*. In addition, we present findings which show that *in vivo* up-regulation of Hsp by carvacrol triggers Hsp-specific Treg that suppress an experimentally-induced inflammatory condition. In summary, we provide evidence for a novel pathway of diet-induced activation of Treg. Additionally, our data show for the first time that the immune system can actually sense altered expression levels of stress proteins and that this can lead to the suppression of an autoimmune arthritis. Given the fact that carvacrol is a normal dietary component, there can be ample and attractive possibilities for the development of carvacrol as a novel preventive or therapeutic drug in rheumatoid arthritis and possibly in other chronic inflammatory diseases.

Materials and Methods

Cell-culture

RAW 264.7 and A20 cell-lines were purchased from American Type Culture Collection. CTLL-16 (37) was obtained from Sanquin Blood Bank. $CD4^+$ T cell hybridomas were generated in our laboratory. In brief, BALB/c mice were immunized with an immunodominant mouse Hsp70 peptide (Hspa1a amino acid positions 169 to 183) or PG peptide (amino acid positions 70 to 84). Subsequently, isolated spleen cells were fused with BW5147 fusion partner and seeded into 96 wells plates at one cell per well followed by analysis of peptide specificity of the obtained clones (38) and (chapter 3). The Hsp70-specific hybridoma was cross-reactive with the homologous peptide in mycobacterial Hsp70. RAW264.7 cells were routinely cultured in DMEM supplemented with 5% FBS (Bodinco B.V.) and 100 units ml^{-1} penicillin and 100 µg ml^{-1} streptomycin (p/s) (Gibco BRL). A20, CTLL-16 and the hybridomas were cultured in Optimem supplemented with 5% FBS and p/s, in a humidified 5% CO_2 atmosphere at 37℃ C. BMDC were isolated from the bone marrow of 9-12 week old BALB/c mice and cultured for 7 days in the presence of 10 ng ml^{-1} GM-CSF (Cytogen) with minor modifications (39) and used on day 8 for in vitro assays. Freshly isolated human PBMC were obtained by ficol (Pharmacia) density gradient centrifugation of heparinised blood and subsequently cultured in RPMI 1640 supplemented with 2 mM glutamine (Gibco BRL), p/s and 10% AB-positive heat-inactivated human serum (Sanquin Blood Bank). For *in vitro* carvacrol treatment cells were incubated at indicated concentrations carvacrol (Sigma) dissolved in ethanol or with ethanol as control. Final ethanol concentration in control or carvacrol cultures did not exceed 0.2%.

Mice and *in vivo* treatment

Female BALB/c mice, purchased from Charles River were housed and fed under standard conditions. Experiments were approved by the Animal Experiment Committee of Utrecht University. Carvacrol dissolved in 100 µl olive oil at indicated concentrations or 100 µl of the vehicle olive oil (Albert Heijn) was administered by i.g. gavage at indicated days. Arthritis was induced in 16-26 week old mice (similar age per independent experiment) using a standard immunization protocol by intraperitoneal (i.p.) injection of human PG (300 µg) and 2 mg dimethyldioctadecylammonium bromide (DDA) (sigma) emulsified in 200 µl PBS on days 0 and 21. Onset and severity of arthritis were determined using a visual scoring system based on swelling and redness of paws (40). For Hsp70 and ovalbumin (OVA) immunization BALB/c mice (9-12 weeks) were i.p. injected on days 0 and 14 with 100 µg recombinant Hsp70 of Mycobacterium tuberculosis (Mt), (LiONEX Diagnostics & Therapeutics GmbH), or 100 µg OVA gradeV (Sigma) and 2 mg DDA, emulsified in 200 µl PBS. To avoid interference of LPS contamination with Hsp70 treatment, Hsp70 containing less than 2.1 EU mg^{-1} protein was used.

Adoptive T cell transfer

Donor BALB/c mice (9-12 weeks) were carvacrol or vehicle treated as described above on days -16, -14, -12 and -9. On day -1, $CD3^+$ T cells from mLN cells were isolated and enriched by negative selection with Dynalbeads (Dynal GmbH) according to manufactures protocol using monoclonal antibodies to: B220 (RA3-6B2), CD11b (M1/70) and MHC class II (M5/114), resulting in a cell suspension containing >98% $CD3^+$ cells. Then, $5x10^6$ $CD3^+$ donor cells, pooled from 5 carvacrol or vehicle treated mice, were i.v. transferred to naïve recipient mice followed by arthritis induction on day 0 and day 21.

Flow cytometric analysis of surface markers, Foxp3 and Hsp70 expression.

Single cell suspensions of spleen or mLN were stained with monoclonal antibodies to CD4 fluorescein isothiocyanate labeled (RM4-5) and CD25 allophycocyanin labeled (PC61) (BD Pharmingen) in PBS plus 2% FBS. Additionally, Foxp3 staining was carried out with a Foxp3-r-phycoerythrin (FJK-16S) staining kit according to manufactures protocol (eBiosciences). For analysis of intracellular Hsp70 expression, cells were fixed and permeabilized for 20 minutes with Cytofix/Cytoperm solution (BD Pharmingen), washed and then incubated with either antibody to Hsp70 fluorescein isothiocyanate labelled (SPA810) (Stressgen) and specifically recognizing the inducible Hsp70 (HspA1A/HspA1B) or the corresponding isotype control in Permwash (BD Pharmingen) supplemented with 2% normal mouse serum. For final analysis of fluorescence a FACS-Calibur (BD Pharmingen) was used.

Analysis of PG-specific serum antibody production

PG-specific serum antibody levels were determined by ELISA as described previously (40). PG-specific antibody levels were calculated as OD relative to the OD measured for the corresponding isotypes of a standard of pooled sera from arthritic mice.

Detection of antigen-specific T cell responses

Single-cell suspensions of spleens were cultured in triplicates in 96-well flat bottom plates at $2x10^5$ cells per well, in the presence or absence of mycobacterial Hsp70 (20 $\mu g\ ml^{-1}$) or OVA (20 $\mu g\ ml^{-1}$) in a humidified 5% CO_2 atmosphere at 37°C. As culture medium IMDM supplemented with 10% FBS, p/s and 50 µM 2-mercaptoethanol was used. After 72 hours, cells were pulsed overnight with [^{3}H]-thymidine (0.4 µCi per well; Amersham Biosciences Europe GmbH), harvested and [^{3}H]-uptake was measured by liquid scintillation counting (Microbeta, Perkin-Elmer Inc.). For analysis of hybridoma activation, A20 B lymphoma cells were either or not exposed to carvacrol (0.2 mM) and heat shock at 42.5°C followed by three hours recovery at 37 °C. Differentially treated A20 cells were cultured with Hsp70- or PG-specific $CD4^+$ T cell hybridomas both at $5x10^4$ cells per well, in 96-wells flat bottom plates. To the indicated cultures mycobacterial Hsp70 (100 $\mu g\ ml^{-1}$), murine $Hsp70_{(169-183)}$ or $PG_{(70-}$

$_{84)}$ peptides (0.005 µg ml^{-1}) were added. After 24 hours, harvested supernatants were cultured with IL-2 responder CTLL-16 cells for 24 hours, pulsed overnight with [^{3}H]-thymidine followed by analysis of incorporation.

Analysis of mRNA expression by quantitative Real-Time PCR

Total mRNA extraction, with the RNeasy kit (Qiagen Benelux B.V.), on column DNAse treatment (Qiagen Benelux B.V.), and transcription into cDNA using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratries B.V.) were carried out according to manufacturer's protocol. PCR (3 min at 95 ºC and 40 cycles of 10 s 95 ºC and 45 s at 59.5 ºC) and Real-Time detection were performed in a Bio-Rad MyiQ iCycler (Bio-Rad Laboratries B.V.). Amplification was done using IQ™ SYBR Green® Supermix (Bio-Rad Laboratries B.V.) with 0.25 µM final concentrations of primers specific for IL-10 (5'-GGT TGC CAA GCC TTA TCG GA-3'and 5'-ACC TGC TCC ACT GCC TTG CT-3'), Foxp3 (5'- CCC AGG AAA GAC AGC AAC CTT-3' and 5'-TTC TCA CAA CCA GGC CAC TTG-3'), CD4 (5'-GAC TGA CCC TGA AGC AGG AG-3' and 5'-CTG GTT CAC CCC TCT GGA TA-3'), Hsp70 (5'-AAG AAC GCG CTC GAA TCC TA-3' and 5'-GAG ATG ACC TCC TGG CAC TTG T-3'), and hypoxanthine-guanine phosphoribosyltransferase (HPRT) (5'-CTG GTG AAA AGG ACC TCT CG-3' and 5'-TGA AGT ACT CAT TAT AGT CAA GGG CA-3'). For each sample mRNA expression was normalized to the detected Ct value of HPRT.

Statistical analysis

Statistical analysis was carried out using Prism software (version 5.00, Graphpad Software Inc.). Significance level was set at ($p < 0.05$). For in vitro analysis of Hsp70 expression (Figure 2) and hybridoma activation (Figure 4A) one way ANOVA (two tailed) with Bonferroni correction was used. Significant differences in the other experiments were determined using Man Whitney U test (two tailed).

Results

Carvacrol amplifies Hsp70 expression induced by *a bona fide* stress response

As the effect of carvacrol on stress protein expression in eukaryotic cells has not been studied before, we studied its effects *in vitro* on various mammalian cell types: bone marrow derived dendritic cells (BMDC) and RAW264.7 macrophages from BALB/c mice, and human peripheral blood mononuclear cells (PBMC). We started measuring the outcome of *in vitro* carvacrol incubation on inducible Hsp70, as Hsp70 is one of the most abundantly expressed stress proteins during stress and locally at the site of inflammation (8, 41). At first, we tested whether carvacrol treatment could directly induce Hsp70 by exposing the cells overnight to either carvacrol, its vehicle ethanol (0.2%), or medium alone. Incubation with carvacrol did not enhance Hsp70 expression as compared to medium or vehicle exposed cultures (Figure 1A). Also at higher concentrations (1 mM and 0.5 mM) carvacrol did not increase Hsp70 levels (data not shown). This showed that carvacrol does not cause cell stress by itself.

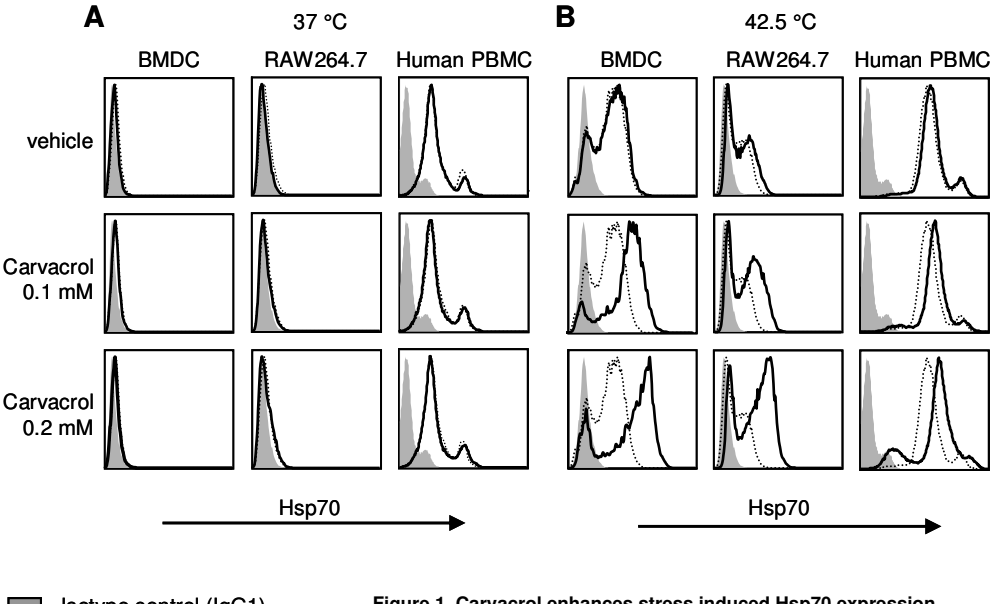

Figure 1. Carvacrol enhances stress induced Hsp70 expression.
(A) Mouse bone marrow derived dendritic cells (BMDC), RAW264.7 macrophages and human PBMC were incubated overnight at 37°C with carvacrol at 0.1 mM or 0.2 mM or with vehicle (ethanol at 0.2%) followed by analysis of intracellular Hsp70 expression by flow cytometry. (B) Cells were incubated with carvacrol or vehicle for two hours at 37 ºC, followed by one hour heat shock at 42.5°C. After overnight recovery at 37 ºC, intracellular Hsp70 levels were analyzed.

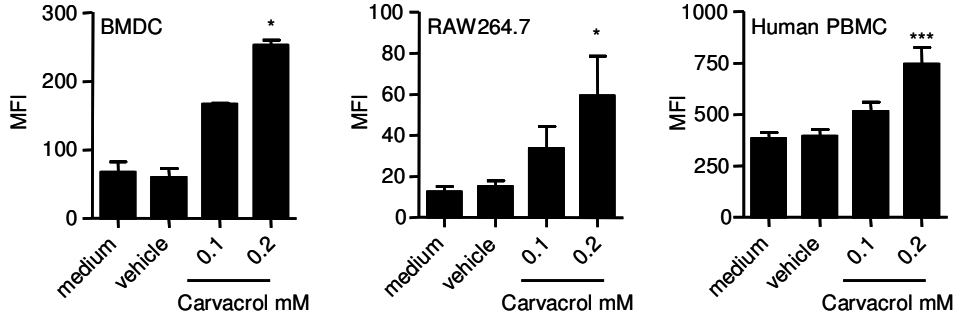

Figure 2. Carvacrol enhances stress-induced Hsp70 expression. Mean fluorescence intensity (MFI) of $Hsp70^{+}$ cells from figure 1B. Data show means (± s.e.m.) of at least three independent experiments (BMDC and RAW264.7) or for 6 donors (PBMC). Significant differences of medium control compared to vehicle or carvacrol exposed cells, are indicated by * ($p<0.05$) and *** ($p<0.001$).

Some other compounds have been reported, however, to amplify Hsp expression induced by *a bona fide* stress response rather than inducing Hsp themselves (36, 42). We therefore studied the capacity of carvacrol to co-induce Hsp70 expression, by two hours incubation at 37°C, immediately followed by a classical stress inducer, i.e. one hour heat shock at 42.5°C. After overnight recovery at 37°C, we measured Hsp70 levels, and found that pre-incubation with carvacrol enhanced heat shock induced expression of Hsp70 in all three cell types (Figure 1B, 2). This was also found in BMDC of mice after 6 hours of recovery (Figure 3). Furthermore, by testing an extended range of carvacrol concentrations we found 0.2 mM as the optimum concentration for maximum Hsp70 co-induction (data not shown). Thus, in several cell types carvacrol is a potent enhancer of stress induced Hsp70 expression.

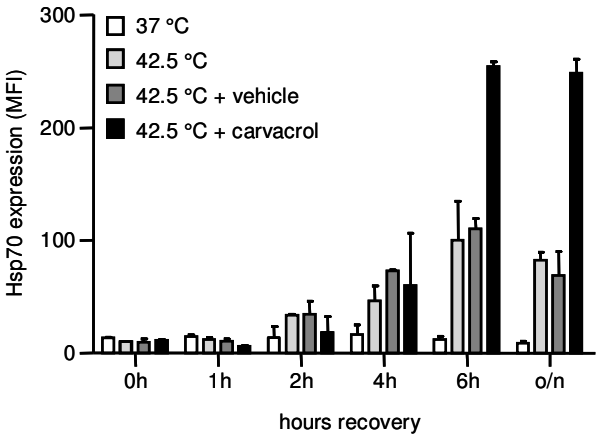

Figure 3. Carvacrol amplifies stress-induced Hsp70 expression. Bone marrow derived dendritic cells (BMDC) were incubated with or without 0.2 mM carvacrol or vehicle only (ethanol at 0.2%) for two hours at 37°C followed by one hour of heat shock at 42.5°C while control cells were kept at 37°C. After recovery for indicated time at 37°C intracellular Hsp70 expression was determined by flow cytometry. Values show mean (± s.d.) fluorescence intensity (MFI) of Hsp70 positive cells of two independent experiments.

Carvacrol enhanced Hsp70 expression activates Hsp70-specific T cells

To determine whether Hsp70-specific T cells can respond to enhanced Hsp70 levels, we studied *in vitro* activation of a $CD4^+$ T cell hybridoma in response to carvacrol treated antigen presenting cells (APC). This hybridoma was generated on the basis of specificity for an immuno dominant Hsp70 peptide, mouse Hspa1a amino acid positions 169 to 183 (Wieten et al. in preparation). We exposed A20 lymphoma cells to carvacrol plus heat shock as in Figure 1B and let them recover for three hours at 37°C, to allow initial up-regulation of Hsp70 and to ensure that Hsp70 expression peaked during co-culture with T cells after the recovery period. Hybridoma activation was measured through IL-2 production as detected by proliferation of the IL-2 dependent CTLL-16 line (37). Hsp70-specific T cells responded well to the addition of Hsp70 peptide and protein to APCs kept at 37°C (Figure 4A), very slightly to APCs after heat shock alone, and very well to heat shocked APCs that had been treated with carvacrol. As a specificity control, we also tested these APCs with the 5/4E8 $CD4^+$ T cell hybridoma (38), which recognizes a proteoglycan (PG) peptide (aa 70 to 84) (Figure 4B). Carvacrol treatment did not enhance 5/4E8 proliferation. These data show that the enhanced expression of Hsp70 by carvacrol under *bona fide* stress conditions, was recognized by T cells specific for Hsp70.

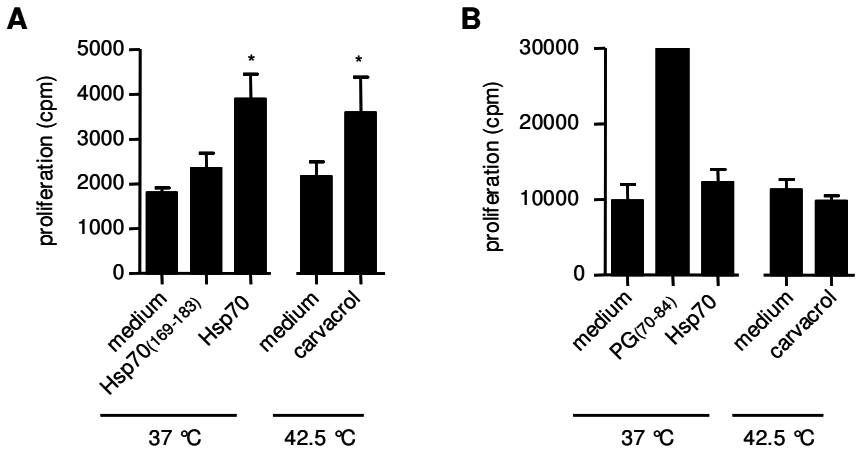

Figure 4. Carvacrol enhanced Hsp70 expression activates Hsp70- but not PG-specific T cells. (A-B) antigen presenting cells (A20 B lymphoma cells) were exposed to 0.2 mM carvacrol for two hours followed by one hour heat shock at 42.5°C. After three hours of recovery at 37°C, A20 cells were cultured with $CD4^+$ T cell hybridomas specific for (A) an immunodominant murine Hsp70 peptide ($Hspa1a_{(169-183)}$) or (B) as a control, a PG peptide ($PG_{(70-84)}$). To some cultures Hsp70, $Hsp70_{(169-183)}$ or $PG_{(70-84)}$ was added. After 24 hours, IL-2 production in the harvested supernatants was determined by the IL-2 dependent CTLL-16. Data are expressed as mean cpm (± s.e.m.) of triplicate cultures. Significant differences as compared to medium control 37°C APC are indicated by * ($p<0.05$).

Carvacrol enhances Hsp70 expression in Peyer's patches and amplifies Hsp70-specific T cell responses

To study the effect of carvacrol on Hsp70 expression *in vivo*, we treated mice by intragastric (i.g.) gavage with either 2, 10, 50 or 200 mg kg^{-1} carvacrol or vehicle (olive oil) as a control on days -6, -4 and -1. Subsequently, on day 0, we determined inducible Hsp70 mRNA expression in cells isolated from spleen, mesenteric lymphnodes (mLN), liver and Peyer's patches. In Peyer's patches, lymphoid tissues located within the intestinal epithelium, and not in other tissues tested, Hsp70 mRNA expression was observed and the expression was augmented up to 500-fold in a dose dependent manner by carvacrol treatment (Figure 5A). Subsequently, we analyzed whether *in vivo* manipulated Hsp70 levels were recognized by Hsp70-specific T cells. Therefore, we pre-treated mice with carvacrol or vehicle on days -8, -6, -4 and -1, followed by Hsp70 or control OVA immunization on days 0 and 14. As expected, Hsp70-specific T cell proliferation was observed in mice immunized with Hsp70 but not in mice immunized with OVA and vice versa (Figure 5B). Interestingly, administration of carvacrol had not modified OVA-induced proliferation, but it had enhanced Hsp70-specific proliferation in Hsp70-immunized mice. This means that carvacrol pre-treatment specifically primed Hsp70-specific T cell responses. No significant effects of carvacrol administration were observed on OVA- and Hsp70-specific antibody responses (data not shown). Thus, in line with the *in vitro* experiments, the *in vivo* experiments demonstrated that manipulation of endogenous stress proteins specifically amplified Hsp70-specific T cell responses.

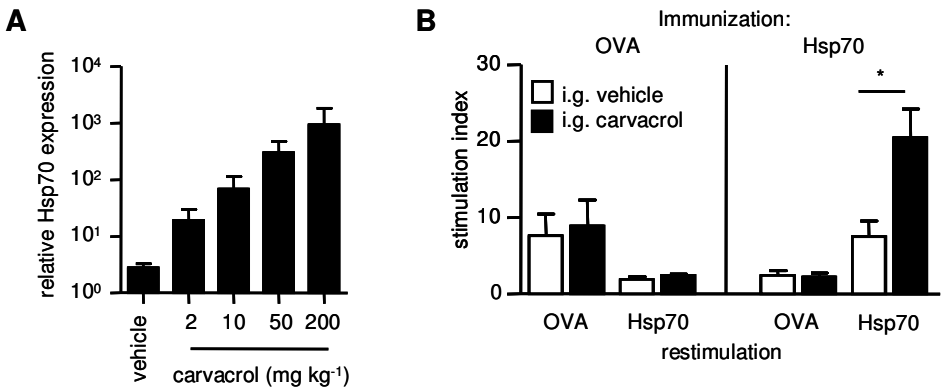

Figure 5. Carvacrol amplifies Hsp70 mRNA expression and Hsp70-specific T cell responses. (A) Mice received carvacrol or vehicle (olive oil) on days -6, -4 and -1 via i.g. gavage. On day 0, Hsp70 mRNA expression was measured by quantitative RT-PCR in Peyer's patches (pooled per animal, four mice per treatment group). Data are expressed relative to HPRT (± s.e.m). (B) Mice were pre-treated with carvacrol (50 mg kg^{-1}) or vehicle by i.g. gavage on days -8, -6, -4 and -1 followed by immunization with Hsp70 or OVA on days 0 and 14. Hsp70- and OVA-specific T cell proliferation was determined on day 28 by detection of [^{3}H]-Thymidine incorporation. Data are expressed as mean (± s.e.m.) stimulation index (mean cpm background/mean cpm antigen). Background cpm levels were 135 (±33) for OVA immunization and vehicle pre-treatment, 364 (±85) OVA-carvacrol, 103 (±21) Hsp70-vehicle and 419 (±135) Hsp70-carvacrol. Statistical difference vehicle compared to carvacrol treated mice is indicated by * (p=0.015) (n=5 per group).

Oral carvacrol treatment suppresses arthritis and increases Foxp3 expression

To explore the capacity of carvacrol to boost immunoregulatory mechanisms, we used the chronic and relapsing model of proteoglycan-induced arthritis (PGIA) (40). Similar to rheumatoid arthritis in humans, PGIA is an autoimmune inflammatory condition caused by both antibodies and pro-inflammatory T cells with specificity for cartilage derived PG. We administered carvacrol or vehicle as described in the previous section and induced arthritis on days 0 and 21. Clinical examination of arthritis symptoms revealed that carvacrol delayed the onset (p=0.02) and reduced severity of arthritis (Figure 6 and Table 1).

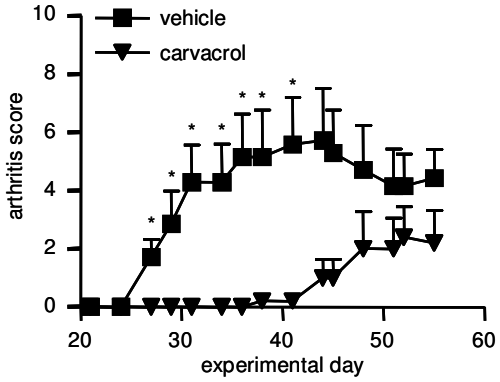

Figure 6. Intragastric carvacrol administration suppresses arthritis. Carvacrol (50 mg kg^{-1}) or vehicle was given by i.g. gavage on days -8, -6, -4 and -1 followed. Then, arthritis was induced by i.p. immunization with PG on days 0 and 21. Arthritis severity expressed as means (± s.e.m.). Data are representative of two independent experiments. Significant differences, carvacrol compared to vehicle treated mice are indicated by * ($p<0.05$) and ** ($p<0.01$) n=7 mice per treatment group).

Table 1. Oral administration of carvacrol suppresses arthritis.

	Incidence	Day of onset	Maximum arthritis score
Vehicle	86%	29.8 (±2.1)	5.4 (±1.1)
Carvacrol	82%	38.5 (±2.8) *	1.6 (±0.5) *

To test the anti-inflammatory capacity of carvacrol, i.g. carvacrol application an dinduction of arthritis were performed as in figure 6. Values indicate means (± s.e.m.) of n= 14 (vehicle) and n=12 (cravacrol) mice per group of two experiments. Statistical differences * ($p<0.05$) comparing vehicle and carvacrol pre-treated groups.

Because of the role of antibodies in PGIA (43), we analyzed PG-specific IgG1 and IgG2a in sera obtained on day 41. Carvacrol administration had not modified PG-specific B-cell responses (Figure 7A-B). This was also observed in sera obtained on days 36 or 55 (data not shown). Because PG-specific IgG1 and IgG2a levels did not differ between the treatment groups, the data suggest that carvacrol had not interfered with disease induction by immunization with PG, but rather with disease progression. Since Treg are important controllers of inflammation (44), we studied the effect of carvacrol on the regulatory T cell associated transcription factor Foxp3 (45). First, we measured systemic Foxp3 expression in

spleen cells. The percentage of $CD4^+CD25^+Foxp3^+$ T cells in the spleen was increased in carvacrol fed mice (Figure 7C). Then, we analyzed Foxp3 mRNA expression at the actual site of inflammation by quantitative RT-PCR analysis of ankle joint derived synovial tissue. Also in the joint, Foxp3 mRNA expression was higher in the carvacrol treated group (Figure 7D). The increased Foxp3 expression, both systemically and at the inflammatory site, indicated induction or activation of regulatory T cells by oral carvacrol administration, a phenomenon that we observed previously after oral Hsp60 treatment (25).

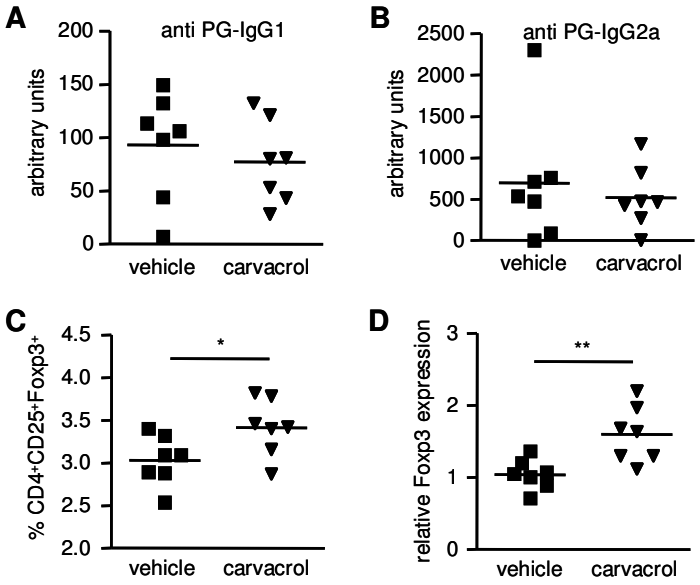

Figure 6. Intragastric carvacrol administration enhances immunoregulation. Carvacrol or vehicle treatment and arthritis induction were done as descried in figure 6. On day 41 serum, spleen and ankle joint synovial tissue were obtained and analyzed: PG-specific IgG1 (A) and IgG2a (B) levels in the sera, expressed relative to a standard of pooled sera from arthritic mice. Data are representative of two independent experiments. (C) Systemic Foxp3 expression was determined in isolated spleen cells by flow cytometry and depicted as percentage $CD4^+CD25^+$ $Foxp3^+$ cells of total viable spleen cells. (D) Local (ankle joint synovial tissue) Foxp3 mRNA expression was measured by quantitative RT-PCR and depicted relative to CD4. * ($p<0.05$) and ** ($p<0.01$) (n=7 mice per treatment group).

Carvacrol induced suppression of arthritis is T cell mediated

To prove the role of T cells in carvacrol-induced suppression of arthritis, we transferred T cells from carvacrol treated mice into naïve recipient mice. We treated donor mice with carvacrol or vehicle on days -16, -14, -12 and -9. On day -1, we isolated $CD3^+$ T cells from the mLN and transferred intravenously $5x10^6$ cells from either carvacrol or vehicle treated donor mice into naïve recipient mice. Then, on day 0, we induced arthritis in recipient mice. Analysis of the donor population showed that carvacrol at this early time point (day -1) had increased the percentage $CD4^+CD25^+Foxp3^+$ cells (Figure 8A) and IL-10 mRNA expression

(Figure 8B) in mLN. Next, in recipient mice the development of arthritis was studied. Adoptive transfer of T cells from carvacrol treated donor mice reduced severity of arthritis (Figure 9 and Table 2). T cell transfer suppressed arthritis almost as effectively as oral carvacrol treatment itself, indicating the presence of carvacrol induced Tregs.

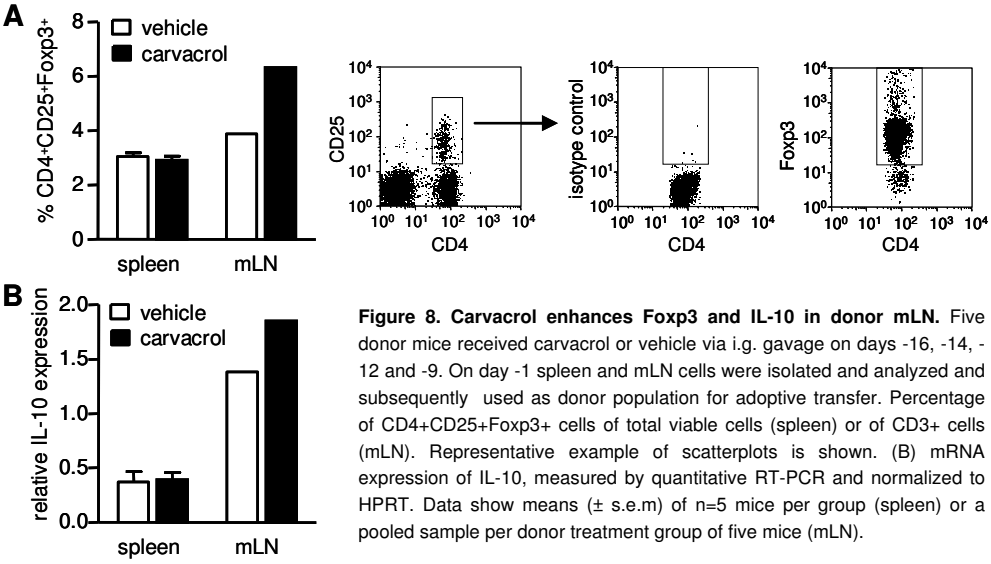

Figure 8. Carvacrol enhances Foxp3 and IL-10 in donor mLN. Five donor mice received carvacrol or vehicle via i.g. gavage on days -16, -14, -12 and -9. On day -1 spleen and mLN cells were isolated and analyzed and subsequently used as donor population for adoptive transfer. Percentage of CD4+CD25+Foxp3+ cells of total viable cells (spleen) or of CD3+ cells (mLN). Representative example of scatterplots is shown. (B) mRNA expression of IL-10, measured by quantitative RT-PCR and normalized to HPRT. Data show means (± s.e.m) of n=5 mice per group (spleen) or a pooled sample per donor treatment group of five mice (mLN).

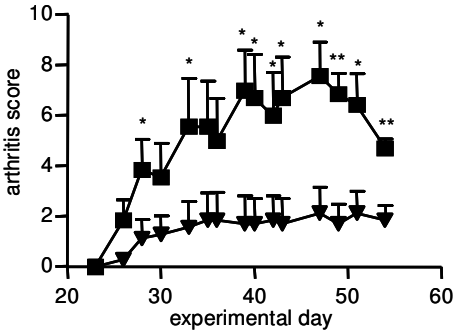

Figure 9. Adoptive transfer of T cells from carvacrol treated donor mice suppresses arthritis. Five donor mice received carvacrol or vehicle via i.g. gavage on days -16, -14, -12 and -9. On day -1 $5x10^6$ $CD3^+$ cells, isolated from mLN, were i.v. transferred to naïve recipient mice. Then, in recipient mice arthritis was induced on days 0 and 21. Data depict arthritis severity in recipient mice expressed as means (± s.e.m.). * ($p<0.05$) and ** ($p<0.01$) of n=7 mice per group. ■ control T cells and ▼ T cells from carvacrol fed donor mice.

Table 2. Adoptive transfer of T cells from carvacrol treated donor mice suppresses arthritis

	Incidence	Day of onset	Maximum arthritis score
Control T cells	100%	27.9 (±1.2)	8.7 (±1.2)
Carvacrol T cells	86%	38.6 (±4.4) *	2.9 (±1.1) **

Adoptive transfer was performed as described in figure 9. Data are expressed as means (± s.e.m.), n=7 per treatment group. Statistical differences * ($p<0.05$) and ** ($p<0.01$) comparing control T cell and carvacrol T cell receiving groups.

Discussion

Inflammation and heat shock are stressful conditions that force cells to up-regulate endogenous heat shock or stress proteins (41, 46, 47). Since the healthy immune system is populated with T and B cells with specificity for self Hsp (48, 49), it has been proposed that the immune system is able to notice changed levels of Hsp expression, leading to an effective immune response as part of a generic damage control mechanism (6, 10). We now report evidence for this by our observation that enhanced expression of stress proteins*, in vivo* induced with the food component carvacrol, was sensed by the immune system and transduced into T cell immunity, leading to suppression of experimental inflammation.

Carvacrol and its isomer thymol are the major components of essential oils obtained from the herbs oregano and thyme. Carvacrol has been mainly been studied as an anti-bacterial agent (34) and from that perspective, carvacrol has been described to increase the expression of Hsp60 but not Hsp70 in prokaryotes (35). The effect of carvacrol on eukaryotic cells however has not been addressed before and we show that carvacrol is a potent enhancer of stress-induced Hsp70 expression in mammalian cells *in vitro*. Because we did not observe an effect of carvacrol in the absence of stress, carvacrol acts as a co-inducer. Previously, other compounds, for example geranyl geranyl actone, have been described to either induce or co-induce Hsp expression depending on the cell type (50). In several cell types, RAW264.7 macrophages, BMDC and human PBMC (Figure 1), we observed differences in heat shock sensitivity and expression levels of subsequently induced Hsp70. However, in none of the cell types we saw an effect of carvacrol on Hsp70 expression in the absence of stress. Administration of Hsp inducers can be expected to constitutively increase stress protein levels, while inducibility is an important hallmark of the protective stress response. Co-inducers on the other hand, will amplify the natural stress response only in the endangered cells and tissues that require Hsp expression. Given the broad array of cellular and immune responses to elevated Hsp levels, a co-inducer may be preferred when applied systemically.

Intragastric administration of carvacrol to mice dose dependently enhanced cellular expression of Hsp70 in Peyer's patches and selectively amplified T cell responses to Hsp70. As carvacrol has been described to penetrate into tissues (51), infiltration in Peyer's patches is likely, subsequently leading to enhanced Hsp70 levels. Peyer's patches are lymphoid tissues, located within the intestinal epithelium and important for mucosal tolerance against orally encountered antigens. Moreover, induction of functional Tregs in Peyer's patches and mLN upon oral application of OVA in WT BALB/c mice has been described before (52, 53). In the normal immune system T cells specific for self Hsp are present (48, 49) and in our study boosting cellular Hsp70 levels in Peyer's patches must have caused increased presentation of Hsp70 peptides on APC, followed by activation or expansion of Hsp70-

specific auto reactive T cells. Our observation that immunization-induced Hsp70- but not OVA-specific T cell responses were enhanced by carvacrol treatment illustrates that the effects observed on T cell immunity were Hsp70-specific. Using an Hsp70-specific T cell hybridoma, we confirmed that carvacrol promotes T cell recognition of endogenous stress-induced Hsp70. In a previous study we showed that enhanced Hsp60 expression in APC, after one hour culture at a raised temperature (43℃), increased the activation of Hsp60-specific T cells (54). These studies are compatible with increased processing and presentation to T cells, as a result of up-regulation of endogenous stress proteins.

Carvacrol administration to mice almost completely suppressed PGIA, a progressive T cell dependent, antibody-mediated murine model for RA (43). In several experimental rodent arthritis models disease is induced with an adjuvant that contains mycobacterial compounds; for example Freunds complete adjuvant. Because Hsp are highly conserved, interfering immune responses, induced by mycobacterial Hsp present in the adjuvant, may have occurred in those models. In the PGIA model we can however exclude such interfering responses as disease is induced by immunization with proteoglycan in the synthetic adjuvant dimethyldioctadecylammonium (DDA). Along with disease suppression, we observed increased numbers of $CD4^+CD25^+Foxp3^+$ T cells both systemically and at the inflammatory site. This suggested that carvacrol co-induced Hsp70 expression had caused an expansion of Treg with the potential to migrate to the site of inflammation. Because Foxp3 is not an activation marker of Treg, increased $CD4^+CD25^+Foxp3^+$ T cell numbers do not necessarily mean increased Treg activation. However, we were able to transfer protection against arthritis with T cells isolated from mLN, where we had seen the local presence of Foxp3 positive T cells of carvacrol-fed mice. Therefore, we reasoned that the immunomodulatory effects of carvacrol were T cell mediated and resulted from activation of carvacrol-induced Treg.
There is the theoretical possibility of other factors involved, such as the induction of microbial stress proteins (Hsp60) at the level of the gut microbiota. Cross talk between stress protein-specific regulatory immune responses has been shown before; vaccination with DNA constructs expressing Hsp70 and Hsp90 suppressed adjuvant induced arthritis in rats. Besides T cell responses to Hsp70 or Hsp90, Hsp60-specifc responses were seen upon vaccination with the constructs (55). Therefore, it will be interesting to study the effect of carvacrol on Hsp60 expression and Hsp60-specific T cell responses and test if up-regulation of one or more endogenous stress proteins leads to activation of cross reactive regulatory mechanisms.

Since Hsp are important in controlling cell damage inflicted by damaged intracellular proteins, the protective capacity of drug induced Hsp expression, aiming at enhanced cellular resistance, has been addressed in several other studies (56). However, the link between targeting stress protein mediated protective intracellular responses and resulting immune-responses has not been studied before. The cellular protective effects of carvacrol

have been addressed previously in TNBS induced colitis; a mixture of essential oils containing carvacrol, decreased pro-inflammatory cytokine mRNA levels and colonic tissue damage in TNBS-induced colitis, but, Hsp specific immune responses were not measured in that study (57).
The cellular pathways through which carvacrol may exert its co-inducing effect on Hsp70 expression are unknown. A possibility is interference with repressor molecules of heat shock factor 1 (HSF1), leading to translocation of HSF1 to the nucleus, binding to Hsp promoter region and transcription of inducible Hsp genes (56). Apart from this carvacrol has been described to inhibit arachidonic acid induced platelet aggregation (58). Similar to what has been seen for other compounds, like NSAIDS and curcumin, carvacrol mediated inhibition of arachidonic acid metabolism may also activate the heat shock response (59, 60). Also, it has been shown that carvacrol may activate the heat-sensitive vanilloid receptor TRPV3 (61, 62) and also mitogen-activated protein kinases JNK and ERK (63, 64). Using an Hsp promoter-luciferase reporter system in the presence of activation inhibitors of these proteins and arsenite as stress inducer, we did not find evidence for their involvement in the Hsp70 co-inducing activity of carvacrol (data not shown).

In several studies stress proteins were shown to have both immune stimulating and immune down-regulating activities (11, 12, 18, 65). Also, medicinal application of stress proteins has been twofold: on one hand to produce effective immune responses to tumors (13) and to serve as carrier molecules for conjugate vaccines (66, 67), while on the other hand to suppress inflammation in various autoimmune diseases, both experimental and clinical (15, 68, 69).
Knowing the prominent immunological activities of stress proteins one could have anticipated the immune system to be responsive to the expression of stress proteins by cells and tissues in situ. In this study we have obtained evidence for this. Because we have shown that the enhanced expression of stress proteins leads to activation of Treg that inhibit autoimmune arthritis, our findings seem especially relevant for the control of inflammation, possibly in both a preventive and therapeutic manner. Also, this may be relevant for the aging individual, since stress inducibility of stress proteins becomes impaired with aging (31, 32, 70). In this manner, the carvacrol boosted expression of stress proteins may assist functioning of Treg in the elderly, who are prone to inflammatory conditions. Since manipulation of inflammatory disease predisposition by dietary means will enable safe and easy intervention, it is attractive to speculate that this intervention can be an adjunct to other currently successful biologicals, such as TNFalfa inhibitors (71, 72). In the adjuvant arthritis model a synergistic effect has been seen by combination treatment with the Hsp60 peptide (180-188) and anti-cytokine therapy, completely suppressing arthritis at lower anti-cytokine dose (73). Such combination therapy, that allows a lower dose of the cytokine inhibitors could be attractive (74).

In conclusion, we show that carvacrol obtained from the herb oregano is uncovered as a powerful co-inducer of stress proteins, active through the mucosal route of administration. Given the nature of carvacrol as a regular food constituent, our findings may add to a rapid and safe development of this compound as novel, orally active, anti-rheumatic drugs. In addition carvacrol and related compounds may be used for development of novel functional foods or neutraceuticals that may support immune regulation by facilitating the cellular production of stress proteins.

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CHAPTER 8

General Discussion

Immunomodulatory mechanism of Hsp-specific T cells

Heat shock proteins (Hsp) have several characteristics that make them relevant targets of the immune system. Receptors for Hsp can be found on cells of both the innate and the adaptive arm of the immune system. Innate receptors are expressed on most immune cells, and include toll like receptors, CD14, CD40, CD91 and CCR5 (1-4). In addition, B and T cell receptors mediate the effects of Hsp on cells of the adaptive immune system. Triggering of the various Hsp receptors can lead to a broad array of immune responses, that can be either immunostimulatory or regulatory (5, 6). Furthermore, Hsp are immunodominant proteins, which has been uncovered by analysis of antigen-specificity of T cell responses induced by immunization of mice with *Mycobacterium tuberculosis* (Mt) and in human leprosy patients. In these studies 10-20% of the T cells recognized Hsp60 of *Mycobacterium tuberculosis* or Hsp70 of *Mycobacterium leprae* (7, 8). Such mycobacterial-Hsp-specific T cell responses have also been observed in healthy individuals, not previously exposed to mycobacterial infections (9) and in cord blood (10). This could be explained by the fact that Hsp are highly conserved proteins (11-13). Therefore, T cells specific for self-Hsp can cross-react with bacterial-Hsp and *vice versa*.

Hsp as immunomodulatory agents

The initial idea that bacterial-Hsp could provoke autoimmunity through molecular mimicry with self-proteins was challenged by the finding that pre-immunization with bacterial-Hsp protected Lewis rats from adjuvant-induced arthritis (AA) (14). Subsequently, we and others showed the immunoregulatory features of Hsp in various inflammatory diseases but, how Hsp exert its regulatory role remained partly elusive. Cohen proposed that to avoid excessive immune responses to both self- and foreign-antigens some immunodominant proteins are being used to monitor and balance the immune system; the immunological homunculus (15, 16). Hsp have been postulated to be such proteins. As will be discussed below, the fact that Hsp are highly conserved proteins is important for suppression of inflammation by Hsp. However, the regulatory capacity of Hsp can not be fully explained by immunodominance and homology between bacterial- and self-Hsp. This was demonstrated in the adjuvant-induced arthritis model, were Hsp was found to suppress disease development while this was not observed with other conserved immunogenic proteins of bacterial origin (17). As argued in chapter 2 up-regulation of Hsp in virtually every cell in response to stress will be an additional essential feature of Hsp.

The concept of Hsp-specific T cell regulation

Hsp have been described to suppress inflammatory disease in a great diversity of models for different immune disorders summarized in (18). This suggested a general immune suppressive mechanism rather than a disease- or model-related suppression. As shown in

transfer studies, Hsp-specific T cells can pass on the immunoregulatory effects (19, 20). Several mechanisms have been proposed for induction of Hsp-specific regulatory T cells (Treg) under physiological conditions (Figure 1) (18, 21). First, bacterial-Hsp-specific Treg can be induced by continuous encounter of bacterial-Hsp, at mucosal surfaces such as the gut. These T cells can be directed towards a regulatory phenotype, through mechanisms of mucosal tolerance. Second, self-Hsp will be up-regulated in response to various forms of stress in almost every tissue. Subsequently, presentation of Hsp-peptides on Major Histocompatibility Complex (MHC) in the absence of co-stimulation, will lead to anergy and possibly to Treg induction and maintenance of Treg. Being not completely identical to their bacterial homologue peptides, such presented self-peptides can also function as altered peptide ligands for bacterial-Hsp-specific cells leading to induction of a partially agonistic T cell response (22). Finally, this induction of Treg might be reinforced by the increased levels of the immunoregulatory cytokine IL-10, induced upon stress in multiple tissues (23).
During inflammation, Hsp will be increasingly expressed and presented on professional antigen presenting cells (APC) at the inflammatory site. At this point, peptide presentation will occur in the presence of co-stimulation, which will lead to full activation of Hsp-specific Treg. In that way activation of Treg can function as negative feedback mechanism, locally dampening ongoing inflammation.

Phenotype of Hsp-specific Treg

The phenotype of Hsp-specific Treg has not been studied in detail. However, since Hsp-specific T cells have been observed in cord blood, some of them will probably be thymus derived $CD4^+CD25^+Foxp3^+$ natural Treg (24, 25). Also, Hsp-specific Treg can be induced in the periphery, which potentially leads to induction of several induced Treg subsets. For example, $Foxp3^-$ Tr1 cells, which are induced by repetitive stimulation with antigen in the presence of IL-10 (26, 27). Alternatively mucosal exposure of Hsp can produce Th3 cells, expressing a $CD4^+CD25^+Foxp3^+$ phenotype (28, 29). Or, conversion of naïve $CD4^+CD25^-Foxp3^-$ cells into induced $CD4^+CD25^+Foxp3^+$ can occur in the presence of IL-2 and TGF-β at low levels of pro-inflammatory cytokines (30).
The phenotype of the Hsp-specific Treg may depend on the exposure route, therefore, we studied T cell phenotype under various conditions. Because exclusive Treg markers have not been described yet, we measured expression of Foxp3, the transcription factor highly associated with Treg and important for Treg development and function (25, 31, 32). In chapter 4 we found that, after intraperitoneal (i.p.) immunization with Mt Hsp70 or Ova as a control, $CD4^+CD25^+$ T cells from Hsp70 immunized mice expressed slightly enhanced levels of regulatory cytokine IL-10, but, did not increasingly express Foxp3. In contrast, in a recent study in a mouse atherosclerosis model, we observed after oral Hsp administration increased Foxp3 expression (33). In line with these findings, Foxp3 expression was augmented on $CD4^+$ T cells after both oral and nasal proteoglycan (PG) treatment (chapter 5). We also found enhanced Foxp3 expression, both systemically in the spleen and locally in

the inflamed joint, upon up-regulation of endogenous Hsp70 in Peyer's patches of carvacrol-fed mice (chapter 7). The finding that Foxp3 levels were increased in cells obtained from joint synovial fluid suggested that induced Treg could have actually migrated to the site of inflammation. In chapter 5 we could provide evidence for Treg migration after mucosal tolerance induction with PG. CFSE labeled $CD4^+$ T cells obtained from either PG- or Ova-tolerized donor mice were intravenously transferred to recipient mice in which proteoglycan-induced arthritis (PGIA) was induced. Subsequently, CFSE labeled cells of both treatment groups could be detected in spleen and paw draining lymph nodes of recipient mice. However, only transferred T cells of PG-tolerized mice increasingly proliferated in draining lymph nodes. Furthermore, transferred T cells of PG- but not of Ova-tolerized mice suppressed proliferation of co-transferred naïve PG-specific TCR-transgenic $CD4^+$ T cells. This indicated that the mucosally induced Treg were functional. Trafficking Treg have been described to express a variety of tissue-specific homing molecules such as CCR7, CCR6 and CD103 (34). To study the phenotype of Hsp-specific Treg in more detail, it will therefore be interesting to see if T cells found at the site of inflammation indeed increasingly express tissue homing markers. Although more research and better Treg markers will be required to study the phenotype of Hsp-specific Treg in more detail, our findings indicate that multiple subsets of Hsp-specific Treg can contribute to Hsp mediated immunoregulation.

Suppressive mechanism of Hsp-specific Treg

Hsp-specific Treg will probably use similar suppressive mechanisms as other antigen-specific Treg, like the production of anti-inflammatory cytokines, cell contact dependent suppression or killing of effector T cells and conversion of APC into a tolerogenic state (35). Furthermore, most Treg subsets use IL-10 for suppression (36) and in previous studies, cross-reactive Hsp-specific T cell responses coincided with the production of IL-10 (17, 19). We addressed the role of IL-10 in modulation of PGIA upon i.p. immunization with Mt Hsp70 (chapter 4) and after nasal administration of Hsp70 peptides (chapter 6). Both treatment strategies enhanced Hsp70-specific T cell proliferation and IL-10 production. Mt Hsp70 immunization failed to rescue IL-10 deficient mice from PGIA development. In both wild type and IL-10 deficient mice Hsp70-specific T cell responses were found, but only in wild type mice these responses suppressed arthritis (chapter 4). In addition, increased PG-specific T cell proliferation, IFN-γ and IL-10 production were found in wild type, but not in IL-10 deficient mice. This illustrated that Hsp70 immunization had also modified the PG response. Given the increase in PG-specific IL-10 production it could be that Hsp70-induced Treg generated a tolerogenic micro-milieu, by their cytokine production. Only in the presence of IL-10 this could promote the outgrowth of new Treg with antigen specificities other than Hsp. These findings emphasized that Hsp-specific Treg used mechanisms of infectious tolerance for modulation of inflammation. This has been shown before in transplantation, type-1 diabetes and EAE models (37-39). Besides IL-10, the role of other cytokines associated with regulatory T cells, like TGF-β and IL-35, has not been addressed but might be relevant.

How important is stress induced Hsp expression?

Hsp expression is up-regulated under virtually every inflammatory condition. Also in autoimmune disease this has been reported; enhanced expression of Hsp60 has been shown in synovial and mononuclear cells of juvenile idiopathic arthritis (JIA) patients (40, 41). In addition, increased expression of inducible Hsp70 and heat shock factor 1 (HSF1) has been shown in the inflamed joint of rheumatoid arthritis (RA) patients (42). This has also been seen for BiP, an ER restricted Hsp70 family member (43).

As mentioned before stress-induced Hsp expression has been proposed to be important for induction, maintenance and activation of Hsp-specific Treg. If indeed so, reduced expression of Hsp can be expected to influence Hsp mediated immune homeostasis and therefore might contribute to development of chronic inflammatory diseases. In fact, Hsp70 polymorphisms have been associated with inflammatory or autoimmune diseases such as Crohn's disease (44), Alzheimer's disease (45), pancreatitis (46) and with development of graft versus host disease upon allogenic haematopoietic stem cell transplantation (47). Furthermore, in the adjuvant arthritis model, delayed induction and lower levels of Hsp60 have been observed in highly susceptible Lewis rats as compared to non susceptible Fisher rats (48). Moreover, diminished Hsp70 expression has also been described in BB rats, highly susceptible for development of autoimmune diabetes (49). Similar results have been found in human peripheral blood mononuclear cells (PBMC) from patients with newly diagnosed type-1 diabetes. In that study, decreased stress responses were re-established in patients with longstanding diabetes. So defective Hsp70 induction coincided with beta cell directed inflammatory activity, this was modulated by pro-inflammatory cytokines rather than metabolic factors (50). Thus, according to literature, decreased Hsp expression has been observed in several immune disorders.

In this thesis we addressed the role of stress-induced Hsp expression by augmenting the heat shock response and studied if up-regulation of endogenous Hsp was actually sensed by the immune system. First we showed that manipulation of the stress response by a classical stress stimulus like heat treatment, enhanced the expression of Hsp70 in immune cells both *in vitro* and *in vivo* (chapter 3). Then we showed that $CD4^+$ T cell hybridomas, generated on the basis of Hsp70 peptide specificity, were increasingly activated upon up-regulation of Hsp in the APC (chapter 3 and 7). Similar results were obtained in a previous study were enhanced Hsp60 levels in heat stressed APC were recognized by Hsp60-specific $CD4^+$ T cells (51). Thus, our studies showed that Hsp-specific T cells can respond to altered Hsp levels.

To further amplify stress-induced Hsp70 expression, we tested multiple food-derived compounds for their effect on Hsp70 expression (chapter 3). One of the compounds, carvacrol, was identified as a potent enhancer of stress-induced Hsp70 both *in vitro.*Also *in vivo,* intragastric (i.g.) gavage of carvacrol enhanced Hsp70 expression in Peyer's patches (chapter 7). We used carvacrol to boost the stress response. We were able to demonstrate immune recognition of carvacrol boosted Hsp levels because incubation of APC with carvacrol enhanced Hsp-specific T cell hybridoma activation. Then, we addressed the

immunomodulatory potential of carvacrol *in vivo*. and found that i.g. carvacrol treatment specifically boosted Hsp70-specific T cell responses. Furthermore, we showed that oral carvacrol administration almost completely suppressed PGIA. Increased local (joint) and systemic Foxp3 expression and the finding that adoptive transfer of T cells, isolated from carvacrol treated donor mice, suppressed PGIA were indicative of the induction of Treg. These findings underlined that Hsp can function as biomarkers for inflammation and that the immunesystem can recognize and react on altered expression of these proteins.

Autophagy, loading Hsp peptides on MHC class II

To activate $CD4^+$ T cells, peptides should be presented in the context of MHC class II molecules. Cytosolic proteins, like Hsp70, are by default loaded on MHC class I molecules while extracellular proteins will be presented on MHC class II. Thus, another fundamental question can be raised; how do Hsp peptides end up in MHC class II? The complete distinction between MHC class I and MHC class II loading pathways has been proven incorrect because cytosolic proteins have been eluted from MHC class II and *vice versa* (52). Autophagy has been initially found as a process to sustain metabolic fitness during food deprivation through bulk protein degradation (53). The role of autophagy in the immune system is only now becoming clear (54, 55). Two pathways can result in loading of intracellular peptides on MHC class II. First intracellular proteins can be incorporated in autophagosomes that subsequently fuse with lysosomes for degradation of their cargo (macroautophagy). In addition, cytosolic proteins can be transported via LAMP2-a directly into the lysosome (chaperone mediated autophagy) (52, 56, 57). Recently, the role of autophagy in loading Hsp70 peptides has been described; in human HLA-$DR4^+$ B cells a striking increase of especially Hsp70 peptides was eluted from HLA-DR4 upon induction of autophagy by starvation (58). Autophagy induction coincided with elevated Hsp70 mRNA levels. Excitingly, the most abundantly eluted Hsp70 peptide in that study had the identical sequence as the mouse/human homologue peptide of the C1 peptide we identified as dominant T cell epitope (chapter 6).

Hsp as therapeutic agents

Hsp inducibility is decreased during aging

During aging susceptibility to inflammatory disease seems to increase. Many other processes are involved but reduced stress responsiveness might contribute. Several studies have shown that stress induced Hsp expression is inversely correlated with age in rodents (59, 60) and in humans (61, 62). Also, in aged immune cells, both lymphocytes and monocytes, decreased expression of inducible Hsp70 has been observed (63, 64). Moreover, reduced Hsp70 levels have been found in B lymphoblasts obtained from average aged individuals, while the expression in cells obtained from centenarians equaled the expression in young people (65, 66). Besides Hsp70, decreased expression of other Hsp family members upon stress has also been reported; Hsp27, Hsp32, Hsp60, Hsp90 and Hsp105 (61, 62), suggesting that aging affects the stress response in general.

Enhanced expression of inducible Hsp, in response to stress, is predominantly mediated by HSF1, a transcription factor sustained in a monomeric form in the cytosol by binding to Hsp70 and Hsp90. Upon stress, the increase in damaged proteins will cause dissociation of the chaperones from HSF1, allowing HSF1 trimerization and translocation to the nucleus. Subsequently, binding of HSF1 to heat shock binding elements (HSE), in the Hsp promoter regions, and phosphorylation of HSF1 lead to transcription of Hsp (67, 68). How age affects the stress response is not completely known but decreased HSF1 binding to Hsp promoter regions has been reported (60, 69, 70). An age related decline in HSF1 protein levels was suggested as a possible explanation (60). Alternatively, while total HSF1 protein levels stay constant, numbers of misfolded HSF1 monomers will increase under aging. Consequently, augmented trimerization of HSF1 monomers including such misfolded monomers will decrease the total number of functional HSF1 trimers during aging (59). A third explanation might be augmented inhibitors of HSF1-HSE binding or loss of HSF1 enhancers (71, 72). Irrespective of the exact mechanism, decreased HSF1 binding to Hsp promoter regions will reduce the expression of stress proteins.

Hsp-specific Treg as target for immunotherapy

A declined stress response can have multiple consequences at the level of the cell and the immune system. We hypothesize that decreased stress-induced Hsp expression leads to failure of Hsp-specific immunoregulation and that this might increase the susceptibility to development of immune disorders. This can happen by multiple means (Figure 1); first, the Hsp-specific Treg response itself is malfunctioning for instance as result of decreased T cell numbers or reduced regulatory properties. Second, the Hsp-specific Treg population is normal, both in T cell numbers and phenotype, yet activation of these Treg, during inflammation, is reduced due to decreased expression and presentation of the self-antigen (Hsp). Targeting Hsp-specific Treg, for immunotherapy purposes in inflammatory disease, could be possible by boosting the Hsp-specific Treg or boosting Hsp expression itself.

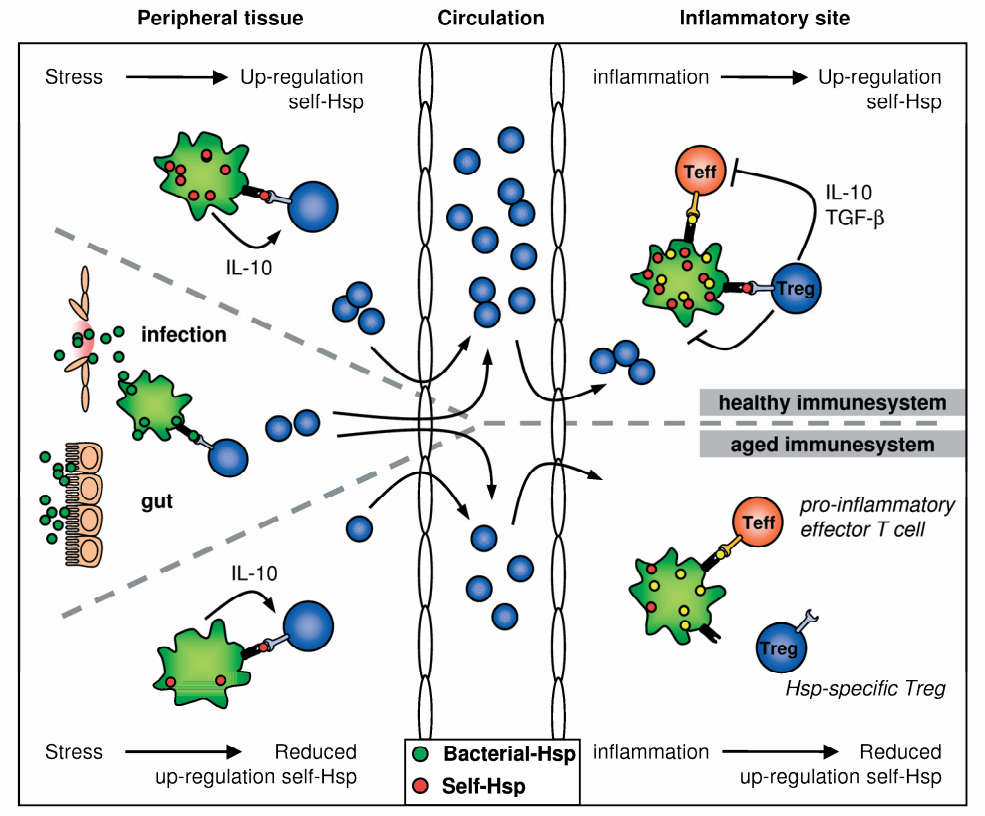

Figure 1. Hsp-specific immunoregulation in the healthy and aged immunesytem.
Self Hsp-specific T cells reside in the circulation after escape from central tolerance in the thymus. Since Hsp are highly conserved these self-Hsp-specific T cells can cross-recognize bacterial-Hsp. This T cell population can be expanded after exposure to bacterial-Hsp at mucosal surfaces like the gut or during infection. At mucosal surfaces, these T cells will be directed towards a regulatory phenotype through mechanisms of mucosal tolerance. In addition, Treg induction and maintenance will be promoted by stress-induced Hsp expression in peripheral tissues, because up-regulation of self-Hsp and presentation of Hsp-peptides on MHC can occur in the absence of co-stimulation. Treg induction will be enhanced by IL-10 produced in response to stress. Furthermore, self-Hsp peptides can function as altered peptide ligands for bacterial Hsp-specific T cells. During inflammation, Hsp will be induced and presented on professional APC at the inflammatory site, leading to full activation of Hsp-specific Treg and local dampening ongoing inflammation. In the aged immune system stress induced Hsp expression is decreased. Therefore, reduced Hsp inducibility will probably influence both the induction of Hsp-specific Treg in the periphery and their activation during inflammation. Ultimately this could result in reduced Treg numbers and function.

Boosting Hsp-specific T cell regulation with exogenous Hsp

In a variety of rodent models of inflammatory diseases, boosting Hsp-specific Treg has been achieved successfully by application of Hsp-proteins or Hsp-derived peptides of bacterial- and self-origin. Hsp60 and Hsp60-derived peptides have been studied extensively, but, immunoregulatory effects have also been observed when other Hsp family members like Hsp70, BiP and Hsp10 were applied, reviewed in (18). Here we focused on Hsp70 and showed that Mt Hsp70 suppressed PGIA upon i.p. immunization (chapter 4) and nasal administration (chapter 6). The immune suppressive effects of Hsp70 have been described before in the adjuvant-induced arthritis model (20, 73). We studied Hsp70-induced suppression of in the mouse PGIA model. This model enabled us to use specific knock out models, like the IL-10 deficient mice described in chapter 4 and the PG-specific TCR transgenic mice used in chapter 5. Furthermore, the PGIA model is a model for chronic and relapsing arthritis with several characteristics shared with human RA. For example PGIA disease development depends on $CD4^+$ T cells and is mediated by B cells and PG-specific antibodies (74). Also, clinical and histopathological features in the PGIA model resemble human RA (75, 76). Moreover, the PGIA model enabled us to study protection in a disease independent manner because arthritis is induced by immunization with PG in the synthetic adjuvant DDA (77). This excludes interfering immune responses between Mt Hsp present in an adjuvant comprised of bacterial components and therapeutically administered Hsp.
To further refine Hsp-mediated T cell therapy, epitope-specific immunotherapy will be an interesting option. Given the broad effects of Hsp on the immune system this could avoid side effects and enables explicit targeting of T cell epitopes involved in disease modulation (78). Therefore, we mapped the immunodominant Hsp70 T cell epitopes (chapter 6) and found that nasal administration of one of the highly conserved peptides C1 or its mouse homologues (mC1a and mC1b) reduced PGIA severity, demonstrating the immunoregulatory potential of the peptide. Additionally enhanced peptide-specific proliferation, IFN-γ and IL-10 production after immunization with the C1 peptide and after nasal administration in PGIA were indicative of boosted peptide-specific T cell responses.

Translation from experimental models to humans

Epitope-specific therapy has proven highly promising in animal models but some fundamental differences between models and humans complicate translation into the clinic. One major difference is the fact that all inbred animals carry the same MHC while the human leukocyte antigen (HLA) system is a highly polymorphic system. We identified the C1 peptide as immunodominant peptide in BALB/c mice, bred on the $H2^d$ background (chapter 6). Peptides for immunotherapy in humans must be recognized by the human immune system and must thus be able to bind molecules of the human HLA system. The C1 peptide is located in an extremely conserved region of the Mt Hsp70 molecule, consequently one of the mouse peptides namely mC1b is completely identical to the human Hsp70. We

addressed peptide binding (*in silico*) to the human HLA class II and found that C1 and the homologues mC1a and mC1b were pan-DR binding peptides (chapter 6). Our findings were strengthened by previous studies showing that Hsp-derived peptides and especially peptides from the C1 region were frequently eluted from MHC class II (chapter 6) and (58, 79). Combined this illustrated that the (m)C1 peptides can most likely bind to various human MHC class II molecules.

Besides peptide binding to HLA, we addressed T cell recognition of the peptides in humans (chapter 6). In healthy controls, T cells recognized the peptide as demonstrated by enhanced proliferation. Furthermore, T cells that recognized the C1 and the mC1b peptide were present in the synovial fluid of the inflamed joint of a JIA and a RA patient. The relevance of such Hsp peptide-specific T cell responses for disease outcome has been demonstrated before by the finding that T cell recognition of human dnaJ peptides correlated with better disease prognosis (80). And in studies showing that Hsp60 or Hsp60 peptide-specific T cell responses were associated with disease remission and a favorable disease outcome in JIA and RA (81-83). Recently, Hsp60-specific T cell responses were also found in juvenile dermatomyositis (JDM) (84). As observed in the synovium of RA patients, muscle expression of Hsp60 was found in biopsies from JDM patients. This indicated that also in JDM, Hsp60 can be a target for the local immune system. New studies with C1 or homologue peptides in human autoimmune diseases will be interesting to expand current knowledge on Hsp-specific T cell responses and their role in inflammatory disease. Furthermore, these studies will be essential for advanced development of therapeutic application of Hsp70 or Hsp70 peptides.

Therapeutic strategies for antigen-specific tolerance induction

The exposure route, antigen dose and the presence or absence of an adjuvant can be critical to specifically induce Treg. In animal models, boosting immunoregulation was successful after administration of Hsp60 or Hsp70 via multiple routes; either intragastrical (33, 85, 86), intranasal (73, 87) (chapter 6) or by parenteral immunization (14, 88) (chapter 4). In the clinical setting, especially the mucosal route will be an attractive way to induce antigen-specific tolerance because it allows easy and non invasive delivery and carries low risk of toxicity (89, 90). Moreover, mucosal tolerance is a naturally occurring pathway of Treg induction based on acquiring of tolerance when food is ingested. Exposure to high antigenic-dose will lead to anergy or deletion of responsive peripheral T cells. In contrast, low antigenic dose can result in the induction of antigen-specific Treg (91). For mucosal tolerance induction the precise application route can determine the effective antigenic dose. For example, for nasally administered antigens the dose required for induction of functional Treg will be lower as compared to the oral dose, as degradation of the antigen will probably be less at nasal- as compared to oral-mucosal surfaces. This has been shown before (92, 93) and also in our study a 10 fold lower nasally applied PG dose, compared to oral administration, efficiently induced tolerance (chapter 5). In line with our study in chapter 5

mucosal administration of disease inciting antigens or peptides has been demonstrated to successfully suppress induction of experimental autoimmune disease (28, 86, 94). Unfortunately, the results in human clinical trials have been variable thus far (27, 91). Alternatives for mucosal tolerance induction have been studied, such as peptide-coupled-cell tolerance, where infusion of immature dendritic cells loaded with peptides leads to induction of Treg that suppress through bystander suppression. This method of tolerance induction has been successfully tested in animal models (95, 96) but not in clinical trials till now.

As argued earlier, the application route could be decisive for the phenotype and regulatory mechanism of the (Hsp-specific) Treg. Differences in regulatory responses induced or boosted by Hsp or Hsp-peptides between application routes have not been studied extensively but can be relevant.

The relevance of the route of exposure was illustrated in chapter 5 were we explored nasal and oral tolerance to PG. Both routes produced functional Treg that suppressed PG-specific IgG2a, but, only orally induced Treg reduced the levels of IgG1. Also, upon i.p. Mt Hsp70 immunization we found decreased PG-specific IgG2a and unchanged IgG1 levels (chapter 4). B cells are important for RA pathogenesis, through the production of antibodies, activation of T cells and formation of lymphoid structures in the joint (97), thus modulation of the B cell response could enhance therapeutic effects. However, B cells can also be involved in dampening inflammation as they can contribute to a more anti-inflammatory microenvironment by producing IL-10 (98). Hsp60-specific antibodies, have been shown to suppress adjuvant-induced arthritis (99, 100). In the PGIA model, we found high levels of Hsp70-specific IgG after immunization with Hsp70 but we did not study immunoregulatory effects of these antibodies (chapter 4). Induction of antibody-producing B cells was not addressed extensively after oral or nasal Hsp application but will most likely be less pronounced as compared to i.p. immunization with an adjuvant.

It will be interesting to study the effect of various adjuvants on induced Hsp-specific T responses. Previously, cholera toxin (CT) has been described as a mucosal adjuvant with anti-inflammatory properties and the potential to induce Treg and bystander suppression (101). In that way CT conjugated to an Hsp60-derived peptide prevented mucosally-induced uveitis in rats (102). In contrast to the latter study, in the PGIA model Hsp is not associated with disease induction. The use of an adjuvant might be especially relevant for immunotherapy with Hsp peptides because these peptides lack for example the induction of IL-10 production by APC that can be observed with the whole Hsp protein.

In first clinical trials both oral and parenteral Hsp peptide application have been tested. Treatment of RA patients with a dnaJP1, an Hsp40-peptide (103), and subcutaneous injection of newly diagnosed type-1 diabetes patients with the Hsp60-peptide DiaPep277 (104, 105), altered the cytokine profile of peptide-specific T cells from pro-inflammatory to more anti-inflammatory without notable side effects. Future research will be essential to refine immunotherapy with Hsp but, the above mentioned studies in patents underlined the potential of Hsp as therapeutic agent, successful through several application routes.

Boosting endogenous Hsp70 expression

Since peripheral Hsp expression is important for Hsp-mediated immunoregulation, we questioned if, besides vaccination with exogenous Hsp, boosting of endogenous Hsp expression could be effective. And if so, could targeting endogenous Hsp with Hsp inducing compounds offer new therapeutic perspectives (chapter 2)? Such up-regulation of stress-induced Hsp levels would be especially helpful in the aged individual were, due to decreased Hsp, the target antigen of Hsp-specific Treg will be reduced. On the other hand, in individuals with a normal stress response boosting Hsp inducibility might enhance induction and activation of Hsp-specific Treg in the periphery and amplify their regulatory function during inflammation.
As described in chapter 2, various compounds have been found that enhance Hsp expression and for some of these compounds anti-inflammatory capacities have been described, summarized in (106-108). But the effect of boosting the stress response on protective T cell immunity has not been studied. In this thesis we present first evidence that *in vivo* targeting the stress response with an Hsp co-inducing compound actually leads to T cell mediated suppression of inflammation (chapter 7).

Boosting Hsp with food-derived compounds

Compounds that induce Hsp, constitutively up-regulate Hsp expression. On the other hand Hsp co-inducers solely enhance Hsp levels in combination with a *bona fide* stress signal (109). In chapter 2 we discussed several compounds that have already been described as Hsp (co-)inducer. To study these compounds in more detail and to explore the immunomodulatory capacity of new food-derived compounds we used three techniques. First, we measured intracellular Hsp70 expression by flow cytometry, which simultaneously allowed for analysis of Hsp70 levels in defined cell subsets (chapter 3). For subsequent testing of the effects on Hsp70-specific T cell responses we generated hybridoma T cells specific for the immunodominant Hsp70-derived C1 peptide (chapter 3 and 7). And we generated the DNAJB1-luc-O23 reporter cell-line to monitor HSF1 and activation of the DNAJB1 promoter (chapter 3).
By flow cytometry we could efficiently detect enhanced Hsp70 levels after incubation with compounds already known to induce Hsp70 expression like geldanamycin (110) and curcumin (111). Thus, we persisted in screening a panel of homogenates derived from spices, dried fruits, nuts, pulses, cereals and sweets, that were recognized for their anti-oxidant capacities (112). Two of these homogenates, black tea and saffron, were demonstrated to increase Hsp expression in combination with heat stress (chapter 3) thus to act as co-inducers. The exact molecule doing this was not identified thus far. In addition to the above mentioned homogenates, we identified carvacrol, as a novel potent co-inducer of Hsp70 (chapter 7).

Boosting Hsp immunoregulation with carvacrol

Carvacrol and its isomer thymol are the major components of essential oils, obtained by distillation of (dried) plant matter, from the herbs oregano and thyme (113). Thus far, carvacrol has been mainly studied as an anti-bacterial agent (114) and from that perspective, carvacrol has been described to increase the expression of Hsp60 but not Hsp70 in prokaryotes (115). The effect of carvacrol on eukaryotic cells had not been addressed before. We showed that carvacrol did not induce Hsp70 itself but, is a potent enhancer of stress induced Hsp70 expression in mammalian cells *in vitro* (chapter 3 and 7). Carvacrol was studied in more detail in chapter 7 and found to enhance Hsp70 expression in Peyer's patches of carvacrol–fed-mice. Furthermore, carvacrol boosted Hsp- but not Ova-specific T cell responses. Then we studied the disease suppressive potential of carvacrol and found that oral carvacrol administration ameliorated development of PGIA. Since adoptive transfer of T cells, from carvacrol treated donor mice, suppressed PGIA effectively, we reasoned that functional Treg were induced.

Our study provided proof of principle that the immune system can respond to compound induced expression of Hsp, while at the same time it raised new questions. How does carvacrol exactly influence Treg? In chapter 7 we discussed that presentation of Hsp70 peptides on APC in Peyer's patches leads to induction of Hsp-specific Treg. The induction of functional Treg in Peyer's patches has been observed before (93, 116). How do these Treg suppress? Most likely they will apply the same mechanism as other induced Treg. Also, the cellular mechanism through which carvacrol causes up-regulation of Hsp is not known, but, in chapter 3 we found that carvacrol also enhanced the expression DNAJB1 (Hsp40), suggesting that carvacrol acted on the stress response in general.

In addition to the effects observed on Hsp-specific T cell immunity, carvacrol could have acted as a mucosal adjuvant. From that perspective, it will be interesting to study the outcome of carvacrol on the innate immune system. The mucosal adjuvant CT has been described to enhance CD80 and CD86 expression (101), while we saw, in an initial study in primary bone marrow derived dendritic cells incubated with carvacrol, only a very small increase in the expression of co-stimulatory molecules CD40 and CD86 as compared to control cells (unpublished results). Previously, curcumin has been shown to suppress arthritis (117) and to inhibit the immunostimulatory capacity of LPS stimulated dendritic cells via inhibition of LPS-induced NF-κB activation (118). In this study the production of LPS induced IL-12p70 was decreased in the presence of curcumin whereas the production of IL-10 was unaffected. It will be attractive to speculate that carvacrol can modulate APC phenotype however, additional studies will be required to elucidate the effects of carvacrol on the APC.

Since carvacrol is a natural dietary constituent, clinical application of carvacrol seems relatively safe. Furthermore, the finding that carvacrol was effective through the oral exposure route will enable easy administration of the compound, making it a promising candidate for boosting Hsp immunoregulation.

The choice of compound

Given the broad array of Hsp functions, constitutive (over)expression of Hsp could have side effects. Therefore, for systemic application, the use of co-inducers will probably be of particular interest. Co-inducers, like carvacrol will only increase Hsp levels after normal induction of the protein. Thus, co-inducers will restore or amplify a physiological regulatory mechanism, and probably act on both the level of the cell and the immune system. Boosting both cellular and immune protection can be highly wanted because inflammation will also be associated with cellular-stress and cell damage. Many of the known Hsp (co-)inducers have also been described as anti-oxidant (119-121). Also, the anti-oxidant capacity of carvacrol has been described (122). We however, did not find a clear relation between antioxidant capacity of the food extracts we tested *in vitro* and the effect on Hsp70 expression (chapter 3).

As described in chapter 7 carvacrol suppressed PGIA, it will however be highly interesting to study the outcome of Hsp co-inducers in other disease models. Furthermore, we treated mice only prophylacticly with carvacrol (chapter 7). In human autoimmune disease, treatment usually starts after development of disease. It will therefore be relevant to study the effect of carvacrol or other compounds on established disease. In addition, the effect of long term exposure to co-inducers should be considered. Several compounds, described to enhance Hsp expression, have already been used in humans. For example aspirin and geranyl geranyl actone (GGA). The latter has been used as anti-ulcer drug without adverse side effects. In the future, regular food constituents like carvacrol, may provide a novel preventive or therapeutic intervention option in chronic inflammatory disease.

Future perspectives

Hsp expression or Hsp-specific T cell responses have been positively associated with a better disease prognosis (82, 83). In addition, the immunosuppressive action of Hsp has been demonstrated in multiple rodent disease models. So, it is attractive to speculate that simply enhancing Hsp immunoregulation in either way could be used as therapy. Apparently, this is oversimplified. Depending on multiple factors such as disease etiology and inflammatory status, patient age and genetic background, difficulties will be encountered. In general, defects in for example positive or negative selection in the thymus, IL-2 production by effector T cells or IL-10 or TGF-β production by Tregs can lead to loss of peripheral tolerance as a result of decreased T cell numbers or functioning (123). Some of these defects might also influence Hsp-specific Treg. For example our findings that Hsp70-induced suppression of arthritis failed in the absence of IL-10 (chapter 4), illustrated that defects in IL-10 production will also influence Hsp-specific Treg. Furthermore, as disease progresses, severe ongoing inflammation has been described to obstruct the effectiveness of antigen-specific Tregs (124, 125). It is currently not known if Hsp-specific Treg can also be hampered by ongoing inflammation. Recently, it has been reported that natural Treg but not induced Treg can convert into Th17 cells after exposure to IL-6 and TGF-β (30). Besides Th1 cells, Th17 cells are major pathogenic effector cells in many autoimmune diseases. Whether Hsp-specific Treg can convert into Th17 cells has not been studied, but if so, timing and route of boosting the Hsp response could be important to avoid exacerbation of disease in stead of induction of regulation.

Boosting Hsp immunoregulation in the elderly

As the immune system ages several changes can be observed such as, declined production of naïve $CD4^+$ T cells, probably due to decreased thymic output (126). Simultaneously, longer residence of remaining naïve T cells has been suggested, but, these T cells would be less functional and produce a reduced amount of Il-2 upon activation (127). Because Treg require IL-2 for induction and maintenance decreased IL-2 levels could influence the Treg compartment, including Hsp-specific Treg. Moreover, vaccination efficacy has been described to be reduced in elderly people, reviewed in (128, 129). Clearly, such reduced efficacy should also be taken into account when considering vaccination based therapies with Hsp or Hsp-peptides.

At increasing age the thymus involutes and natural Treg numbers will decrease, but induced Treg numbers have been suggested to increase (130). Shifting Treg populations can have implications for Hsp mediated therapeutic interventions in aged individuals as it might influence the choice of adjuvant and application route in order to boost the most potent Treg subset in the aged individual. As discussed earlier, stress-induced Hsp expression is negatively associated with age. Boosting Hsp-specific Treg numbers could therefore be less effective in aged individuals because of reduced expression of the target antigen.

Combination therapy

Combination therapies might offer new opportunities to overcome adverse features of single therapies. In that way Hsp can also be valuable adjuncts to currently established therapies with for example immunosuppressive agents. This has been demonstrated in the rat adjuvant-induced arthritis model were a synergistic effect on disease inhibition has been found by combination treatment with Hsp peptides and TNF-α inhibition (131). Thus, combination therapy will probably increase antigen-specific Treg responses and simultaneously allow lower dose of the immunosuppressive agent. In addition, alternative combination therapies would be interesting (Figure 2).

We postulated that in individuals with decreased stress-induced Hsp expression, Hsp-specific Treg numbers and function can be ameliorated. Combination of boosting T cell numbers, with exogenous Hsp vaccination, and simultaneously enhancing Hsp expression with co-inducers would be attractive. However, increased expression of Hsp will only have an antigen-specific effect on the Hsp-specific Treg response, if this is accompanied by enhanced presentation of Hsp-peptides on MHC class II. Thus, combination of compounds that co-induce Hsp expression and enhancers of Hsp-peptide presentation can at least in theory act synergistically. Alternatively, agents that promote induction or activation of Treg can be combined with co-inducers or Hsp peptide application. In that way, rapamycin has been described as autophagy inducer, and also the increased activation of Treg upon rapamycin has been reported. In summary this illustrates the (potential) synergistic interplay between targeting Hsp immunoregulation through various pathways and therapies currently established or under investigation.

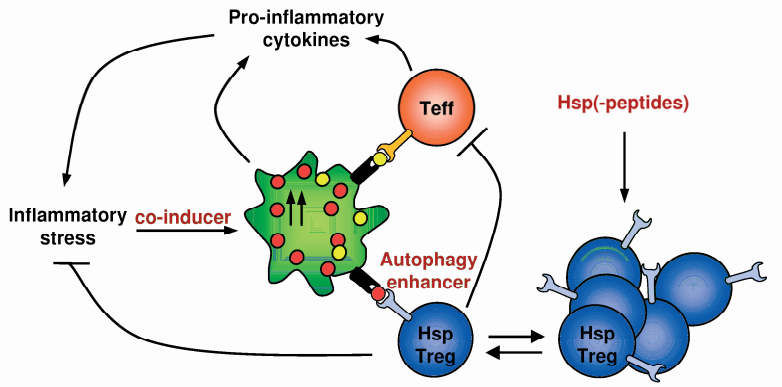

Figure 2. Hsp-specific T cell immunity as target for immunotherapy. Expression of inducible Hsp70 is increased in response to various forms of stress and triggers naturally occurring feedback mechanisms; During inflammation, enhanced Hsp70 levels will amplify immunoregulation by Hsp70-specific Treg and, upon activation, these T cells can dampen ongoing inflammation. Antigen-specific immunotherapy with exogenous Hsp or peptides will boost the number and function of Hsp-specific Treg. On the other hand, boosting endogenous Hsp expression will increase Hsp-specific Treg numbers, function and expression of the target antigen at the site of inflammation. Autophagy enhancers will augment presentation of Hsp-peptides on the MHC class II-leading to enhanced T cell activation.

In conclusion

Hsp are important controllers of cellular and immune homeostasis and are expressed in almost every tissue. Because Hsp expression is directly influenced by both intracellular and extracellular stress signals, Hsp levels reflect the state of the cell and the tissue and can be used to translate stress signals into effector mechanisms. Activation of Hsp-specific Treg is such an effector mechanism and will be important to maintain immune homeostasis. As discussed here various factors influence immune suppressive capacity of the Hsp response. Factors like, the T cell response itself, expression of the Hsp antigen and presentation of Hsp-peptides on MHC class II. Targeting these factors, either or not in combination with established therapies, can be interesting means for antigen-specific immunotherapy to prevent or suppress autoimmune disease. Our findings presented here enhance basic knowledge on how Hsp-specific T cells can modulate the immune system, which will contribute to development of new or refinement of existing intervention strategies to treat autoimmune disease.

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Summary

Summary

Breakdown of immunoregulatory mechanisms can lead to loss of self tolerance and autoimmune disease, coinciding with chronic inflammation and tissue damage. The ultimate target organ will differ per autoimmune disease, but basic features of failing regulatory systems and chronic inflammation will be shared between the various diseases. Many existing therapies in autoimmune disease are based on general suppression of inflammation and the side effects observed with these therapies illustrate the pressing need for more specific interventions. Regulatory T cells (Treg) are pivotal controllers of autoaggressive immune responses and inflammation, and decreased Treg numbers and/or functioning have been associated with autoimmune disease. Therefore, Treg became frequently studied targets for more specific immunotherapy. Especially antigen-specific targeting of Treg would enable local and tailor made intervention.

Self-antigens, that participate in inflammation yet, irrespective of the etiology of the different autoimmune diseases, can be promising candidate antigens for antigen-specific interventions. Rather than tolerance induction to disease inciting self-antigens, that are frequently unknown, these antigens would allow targeting of disease independent but, inflammatory site-specific regulatory pathways. Preferably, such self-antigens should be abundantly expressed at the inflammatory site. Heat shock proteins (Hsp) have several characteristics that can make them highly attractive mediators of antigen-specific therapy. Being stress proteins, Hsp expression is increased during every form of inflammation. This and the fact that Hsp are conserved and immunodominant proteins make them highly relevant targets for the immune system. The regulatory potential of exogenous Hsp has been shown before, both clinically in patients and in experimental models of inflammatory diseases. However, basic knowledge on the mechanisms of Hsp-specific Treg will be indispensable to enlarge and refine knowledge on Hsp as therapeutic agents.

Stress-induced Hsp expression has been proposed by us and others to be essential for proper functioning of Hsp-specific T cell responses. Decreased Hsp inducibility has been observed in aged individuals and in some immune disorders. Assuming that reduced Hsp levels can result in failure of Hsp-specific regulation of inflammation, this may contribute to increased disease susceptibility. Therefore, we hypothesized in this thesis that endogenous Hsp are targets for anti-inflammatory T cells and that boosting either Hsp-specific T cell responses or Hsp expression itself can restore or enhance immune homeostasis. Because especially Hsp70 is a highly stress inducible Hsp family member, we focussed on Hsp70. The aim of the studies described in this thesis was twofold. First, we wanted to further unravel the mechanism of Hsp70-specific T cell regulation. Second, we addressed the potential of Hsp70 as therapeutic agent, by antigen- or epitope specific immunotherapy with exogenous Hsp70 and Hsp70-peptides and via compound induced up-regulation of endogenous Hsp70.

In **chapter 2** we discuss Hsp-mediated immunoregulation in general and question how Hsp-inducing compounds can be exploited to boost regulation. Subsequently, in **chapter 3** we show that Hsp70 is highly stress inducible in immune cells and that we can actually boost Hsp70 expression in immune cells with food-derived compounds. Using flow cytometry, Hsp70-specific $CD4^+$ T cell hybridomas and reporter cell lines, we monitor the effect of food-derived compounds on Hsp expression and Hsp70-specific T cell activation.
Then we proceed to investigate the effect of boosting Hsp-specific T cell responses with exogenous Hsp or Hsp-derived peptides in the proteoglycan-induced arthritis (PGIA model). First, in **chapter 4** we demonstrate that intraperitoneal immunization with mycobacterial (Mt) Hsp70 suppresses PGIA. Moreover, we provide evidence for the mechanism of Hsp-specific Treg by our finding that IL-10 is essential for suppression.
Since mucosal antigen administration will be an appealing way of tolerance induction, we study the cellular basis of mucosal tolerance in the PGIA model in **chapter 5**. We show that both oral and nasal administration of proteoglycan induce Treg that can suppress PGIA and proliferation of arthritogenic T cells in joint draining lymph nodes. Then, in **chapter 6** we confirm that nasal administration of Mt Hsp70 ameliorates PGIA. We also identify the immunodominant T cell epitopes of Mt Hsp70 in BALB/c mice and describe that one of the highly conserved epitopes induces IL-10 and suppresses PGIA upon intranasal administration. We uncover in addition, that this peptide is a good binder (in silico) to the human HLA and is recognized by human T cells.
In **chapter 7** we target Hsp70 expression directly and manipulate Hsp levels with the food constituent carvacrol. We describe that carvacrol is as a very potent enhancer of stress-induced Hsp70 expression both *in vitro* and upon intragastric administration *in vivo*. Up-regulation of Hsp70 is sensed by Hsp70-specific T cell hybridomas and also *in vivo* we confirm that carvacrol enhances Hsp70-specific T cell responses. Subsequently we demonstrate that intragastric administration of carvacrol suppresses PGIA in a T cell dependent manner, thus providing the first evidence that boosting endogenous Hsp can be translated into immune regulation.
In summary the findings presented in this thesis show that boosting Hsp70 expression or the Hsp70-specific T cell response can modulate the immune system and enhance immune homeostasis, thereby illustrating that endogenous Hsp can indeed function as targets for anti-inflammatory T cells.

Nederlandse Samenvatting

Inleiding

Het immuunsysteem van zoogdieren is een ingewikkeld netwerk met verschillende soorten immuuncellen (witte bloedcellen of leukocyten), ieder met een eigen functie. Door samenwerking tussen de leukocyten beschermt het immuunsysteem het lichaam tegen ziek makende indringers (pathogenen) zoals virussen en bacteriën en tegen tumorgroei. Het immuunsysteem kan grofweg in twee takken onderverdeeld worden: Het aangeboren immuunsysteem wordt gekenmerkt door een snelle maar niet heel specifieke reactie en is vooral belangrijk als eerste bescherming tegen indringers. Karakteristiek voor het specifieke immuunsysteem zijn specificiteit en geheugen.
De cellen die een belangrijke rol spelen bij de specifieke immuunrespons herkennen bepaalde structuren (antigenen) op bijvoorbeeld een pathogeen en kunnen hier vervolgens op reageren. Zogenaamde B cellen en T cellen zijn belangrijke spelers van het specifieke immuunsysteem. Via speciale receptoren (B en T cel receptoren) kunnen zij bepaalde antigenen herkennen en worden daarom ook wel antigeenspecifieke cellen genoemd. Na herkenning van hun antigen kunnen zij meedoen aan het opruimen van pathogenen door bijvoorbeeld antilichamen te produceren of door geïnfecteerde cellen te vernietigen. Daarnaast blijft een deel van de B en T cellen achter waardoor bij een volgende ontmoeting het pathogeen snel en efficiënt opgeruimd kan worden (geheugencellen).

Het immuunsysteem moet dus adequaat kunnen reageren op mogelijke pathogene lichaamsvreemde antigenen maar tegelijkertijd moet worden voorkomen dat het daarmee onnodig schade aan het eigen lichaam toebrengt. Verder moet het immuunsysteem in principe “tolerant” zijn voor lichaamseigen antigenen. In het geval van een auto-immuunziekte bijvoorbeeld, is deze tolerantie verbroken en vindt een agressieve immuunreactie tegen lichaamseigen weefsels plaats. Dus om schade en ongewenste immuunreacties te voorkomen moeten de acties van het immuunsysteem goed gecontroleerd zijn. Hiervoor beschikt het immuunsysteem over een aantal regulatoire mechanismen die er gezamenlijk voor zorgen dat pro- en anti-inflammatoire immuunreacties in balans blijven. In dit proefschrift is met name gekeken naar de rol die T cellen spelen bij het handhaven van die balans.

T cellen zijn van belang voor zowel het opruimen van pathogenen als voor het controleren van immuunreacties. T cellen herkennen, met hun T cel receptor (TCR), stukjes antigeen (peptiden) gepresenteerd op major histocompatibility complex (MHC) eiwitten. De T cel kan zo verschillende antigenen onderscheiden maar kan geen onderscheid maken tussen eigen en niet-eigen antigenen. Om toch tolerantie voor lichaamseigen weefsels te waarborgen, worden T cellen die eigen antigenen herkennen in de thymus verwijderd (centrale tolerantie). Echter dit proces is niet volledig en T cellen die eigen eiwitten kunnen herkennen

zijn aanwezig in de circulatie. Om nu een buitensporige immuunrespons tegen lichaamseiwitten te voorkomen is naast centrale tolerantie, perifere tolerantie nodig.
Een bepaalde populatie T cellen, de regulatoire T cellen (Treg), hebben een centrale rol in het reguleren van de immuunrespons, doordat zij de acties van andere immuuncellen kunnen controleren of blokkeren. Verschillende subpopulaties Treg kunnen onderscheiden worden en deze kunnen aangeboren zijn, of later geïnduceerd worden in de periferie. Door inductie van Treg in de darm kan bijvoorbeeld een overdreven reactie tegen voedsel voorkomen worden. Deze vorm van tolerantie wordt mucosale tolerantie genoemd. Na activatie kunnen zowel aangeboren als geïnduceerde Treg een immuunrespons onderdrukken door onder andere het produceren van anti-inflammatoire cytokinen (signaal moleculen) zoals IL-10, TGF-β en IL-35.

Ondanks regulatie door centrale en perifere tolerantie mechanismen gebeurt het zo nu en dan dat de tolerantie voor lichaamseigen eiwitten gebroken wordt. Hierdoor kan een ongecontroleerde (agressieve) reactie van het immuunsysteem tegen eigen weefsels optreden met als gevolg auto-immuunziekte als reumatoïde artritis (RA) of type 1 diabetes. Het aangetaste orgaan zal per auto-immuunziekte verschillen maar het onderliggende mechanisme van falende regulatie met daardoor een chronische ontsteking is bij veel auto-immuunziekten aanwezig. Als therapie worden patiënten nu vaak met algemene anti-inflammatoire middelen behandeld. Alhoewel deels succesvol, is één van de nadelen dat, door algemene onderdrukking van het immuunsysteem, patiënten gevoeliger kunnen worden voor bijvoorbeeld infecties. Daarom zou een meer specifieke aanpak wenselijk zijn.
Omdat Treg zo belangrijk zijn voor de controle van autoagressieve immuunreacties en defecten in de Treg respons geassocieerd zijn met het optreden van auto-immuunziekten, is al veel onderzoek gedaan naar een mogelijk therapeutisch effect van Treg op auto-immuunziekten. In proefdiermodellen werd gevonden dat Treg tolerantie konden induceren voor het antigeen dat de ziekte veroorzaakt. Daarbij zijn deze Treg vaak specifiek voor ditzelfde antigeen. Helaas blijkt de vertaling van deze proefdiermodellen naar de mens niet zo eenvoudig. Een belangrijke factor hierbij is dat in mensen het antigeen dat de ziekte veroorzaakt vaak niet bekend is, of dat het er meerdere kunnen zijn. Om dit probleem te omzeilen zou mogelijk een Treg gebruikt kunnen worden die een antigeen herkent dat onafhankelijk van de auto-immuunziekte is. Om specifiek op de plaats van ontsteking Treg te kunnen activeren zouden deze antigenen op de plaats van ontsteking tot expressie moeten komen. Daarnaast moeten ze natuurlijk door het immuunsysteem herkend kunnen worden om uiteindelijk tot activatie van Treg en onderdrukking van de ontsteking te kunnen zorgen.

Heat shock eiwitten (Hsp) zijn veelbelovende kandidaat antigenen voor immunotherapie bij auto-immuunziekten. Op basis van hun moleculaire gewicht kunnen Hsp in verschillende families ingedeeld worden, zoals de Hsp60, Hsp70 en Hsp90 families. Hsp hebben een belangrijke rol bij het beschermen van de cel tijdens stress, onder andere doordat zij

belangrijk zijn voor het transport en (her)vouwen van beschadigde eiwitten. De expressie van veel leden van de Hsp families wordt geïnduceerd door stress, zoals oxidatieve- hitte- en ontstekingsstress. Hsp zijn dan ook aanwezig bij iedere vorm van ontsteking.
Hsp zijn sterk geconserveerd tijdens de evolutie waardoor er een sterke overeenkomst (homologie) tussen Hsp van bacteriën en zoodieren is. Het immuunsysteem krijgt te maken met Hsp van verschillende oorsprong. Via ons voedsel of tijdens een infectie komt het immuunsysteem in contact met Hsp van bacteriële oorsprong. Verder komt lichaamseigen Hsp in vrijwel alle cellen van het lichaam voor. Zowel de cellen van het aangeboren als van het specifieke immuunsysteem kunnen op Hsp reageren. In het onderzoek beschreven in dit proefschrift, is met name gekeken naar T cellen die specifiek Hsp herkennen met bovendien een regulerende anti-inflammatoire functie.

De anti-inflammatoire potentie van Hsp-specifieke T cellen werd gevonden in proefdier modellen. Voorbehandeling van ratten, door vaccinatie (immunisatie) met bacterieel Hsp, kon de dieren beschermden tegen het ontwikkelen adjuvant artritis. Nader onderzoek toonde aan dat het inspuiten van Hsp-specifieke T cellen ook de ziekte kon voorkomen. Omdat de T cel alleen bepaalde fragmenten van het Hsp eiwit (epitopen) kan herkennen werd bestudeerd welke epitopen betrokken waren bij bescherming. Hierbij werd gevonden dat ook door behandeling met een sterk geconserveerd Hsp epitoop, ziekte inductie kon worden voorkomen. In eerste instantie werd naar Hsp60 gekeken maar later bleek dat ook andere Hsp, zoals Hsp70 voor inductie van anti-inflammatoire T cellen konden zorgen. Daarnaast had toediening via bijvoorbeeld de orale of de nasale route een zelfde beschermend effect. Ook in andere modellen voor auto-immuunziekten werd bescherming na Hsp behandeling gevonden.
Tijdens een ontsteking worden Hsp opgereguleerd. Ook in het ontstoken gewricht van patiënten met RA of juvenile idiopatische artritis (JIA) is een verhoogde expressie van Hsp60 en Hsp70 gevonden. Hierdoor zouden Hsp locaal voor activatie van Hsp-specifieke Treg kunnen zorgen. Na activatie kunnen deze T cellen de ontsteking reguleren en zo voorkomen dat de ontsteking chronisch wordt. Studies in RA en JIA patiënten toonden aan dat de aanwezigheid van een Hsp-specifieke T cel response geassocieerd is met een gunstiger ziekte verloop.

Dit proefschrift

Inductie van Hsp door stress is waarschijnlijk essentieel voor het goed functioneren van de anti-inflammatoire Hsp-specifieke T cel response. Tijdens veroudering is echter beschreven dat juist die inductie van Hsp verlaagd is. Het zou kunnen dat die verminderde Hsp inductie leidt tot falen van Hsp-specifieke immuunregulatie en dat dit bijdraagt aan de verhoogde gevoeligheid voor het ontwikkelen van auto-immuunziekten op hogere leeftijd. Het falen van Hsp-gemedieerde immuunregulatie kan het gevolg zijn van een minder functionele Hsp-specifieke T cel respons, door een verlaagd aantal Hsp-specifieke T cellen of doordat deze T cellen minder anti-inflammatoir zijn. Aan de andere kant kan het ook zo zijn dat de T cel respons weliswaar normaal is maar niet voldoende geactiveerd wordt doordat Hsp niet of minder tot expressie komen tijdens ontsteking.
Het herstellen of verbeteren van immuunregulatie door Hsp zou dan ook op 2 manieren plaats kunnen vinden: Door het boosten van de T cel respons zelf. Dit kan gedaan worden door bijvoorbeeld het toedienen van Hsp of Hsp-peptiden. Een ander mogelijkheid is het verbeteren van de Hsp induceerbaarheid met Hsp co-inducers. Beide opties zijn onderzocht in de studies beschreven in dit proefschrift. Het doel daarbij was meer inzicht te krijgen in het mechanisme van Hsp-specifieke immuunregulatie. Daarnaast hebben we de mogelijkheid onderzocht om Hsp70 te gebruiken voor antigeen-specifieke immunotherapie.

In hoofdstuk 2 wordt immuunregulatie door Hsp in het algemeen behandeld. Daarnaast wordt bediscussieerd hoe stoffen die voor Hsp opregulatie zorgen gebruikt zouden kunnen worden om het immuunsysteem te moduleren. Een aantal stoffen, waarvan in de literatuur beschreven is dat ze Hsp verhogen, worden kort behandeld. Deze stoffen kunnen in twee categorieën verdeeld worden: de Hsp inducers, welke zelf voor opregulatie van Hsp zorgen en de co-inducers, dit zijn stoffen die zelf geen effect op Hsp expressie hebben maar in combinatie met een extra stress signaal, zoals b.v. hitte stress, wel voor een stijging in Hsp expressie zorgen.

Vervolgens zijn, in hoofdstuk 3, een drietal testsystemen ontwikkeld waarin het effect van bekende en nieuwe, potentiële co-inducers van Hsp onderzocht kan worden, op een manier die ook voor het immuunsysteem relevant is. Eerst is gekeken naar Hsp expressie in immuuncellen. Hierbij werd gevonden dat Hsp70 sterk geïnduceerd werd na zowel *in vitro* als *in vivo* hitte stress. Hsp60 daarentegen bleef onveranderd. Met behulp van flow cytometry kon inductie van Hsp70 efficiënt aangetoond worden in verschillende celtypen. Het voordeel van deze techniek is dat het snel is en dat Hsp expressie door dubbelkleuring in individuele cel populaties bestudeerd kan worden. Vervolgens is een panel extracten getest dat geïsoleerd was uit voeding. Hierbij vonden wij dat extracten van zwarte thee en saffraan co-inducers van Hsp70 zijn. Ook carvacrol, één van de belangrijkste bestanddelen van essentiële oliën uit de kruiden oregano en tijm, werd als co-inducer geïdentificeerd. Een

verhoogde Hsp70 expressie zal alleen een effect op de Hsp70-specifieke T cel respons hebben als Hsp-peptiden ook gepresenteerd worden op MHC en dus “gezien” kunnen worden door de T cel. Om te testen of dit het geval is, na behandeling met de co-inducers, zijn $CD4^+$ T cel hybridoma’s gemaakt die specifiek een immunodominant Hsp70 peptide herkennen. Wij vonden dat deze hybridoma’s inderdaad konden reageren op carvacrol geïnduceerde Hsp70 verhoging. Als derde werd een DNAJB1-luc-O23 reporter cellijn gemaakt om aan te tonen dat carvacrol de activiteit van een heat shock promoter verhoogt. Samenvattend zijn deze testsystemen algemeen toepasbaar om stoffen te identificeren die een effect hebben op Hsp expressie in immuuncellen.

Om de Hsp-specifieke T cel respons te verbeteren is in hoofdstuk 4 het effect van behandeling met mycobacterieel (Mt) Hsp70 op een chronische ontstekingsziekte onderzocht. Hiervoor is het proteoglycaan geïnduceerde artritis model (PGIA) gebruikt. In dit muis-model wordt artritis geïnduceerd door twee injecties met humaan proteoglycaan (PG). Na inductie van de ziekte ontwikkelen de muizen een chronische en recidiverende vorm van artritis waardoor het model sterk lijkt op humane RA. Intraperitoneale injectie met Hsp70, 10 dagen voor inductie van de ziekte, verminderde de ernst van de ziekte. Daarnaast werd de Hsp70-specifieke T cel respons verhoogd, wat meetbaar was door toegenomen T cel proliferatie, en de productie van de cytokinen IFN-γ en IL-10. Om meer inzicht te krijgen in het mechanisme van bescherming door Hsp70, werd de behandeling uitgevoerd in IL-10 knock-out muizen. Alhoewel de Hsp-specifieke T cel respons ook in deze dieren geïnduceerd werd, kon in de afwezigheid van IL-10 geen bescherming waargenomen worden. De conclusie is dan ook dat Mt Hsp70 ook in het PGIA model beschermt maar dat IL-10 hiervoor belangrijk is.

Exogeen Hsp zou op verschillende manieren toegediend kunnen worden. De meest aantrekkelijke manier is misschien wel de mucosale route, dus via orale of nasale toediening. Toediening van antigenen via deze route lijkt relatief veilig en makkelijk. Via de mucosale route komt het immuunsysteem continu met allerlei vaak onschuldige antigenen in aanraking. Om te voorkomen dat het immuunsysteem hierop overdreven reageert, is de reactie in principe inductie van tolerantie tegen het desbetreffende antigeen. Mede daarom is de mucosale route zeer geschikt voor de inductie van antigeen-specifieke Treg. In hoofdstuk 5 is daarom de mucosale inductie van Treg in het PGIA model nader bestudeerd na toediening van het ziekte inducerende PG eiwit. Hierbij werd gevonden dat zowel nasale als orale PG toediening de ontwikkeling van artritis kon onderdrukken. T cellen van PG behandelde dieren brachten verhoogd moleculen tot expressie die geassocieerd zijn met Treg (IL-10, TGF-β en Foxp3). De gevonden bescherming kon vervolgens getransfereerd worden naar nieuwe dieren door het inspuiten van T cellen geïsoleerd uit PG behandelde muizen. Wat aantoonde dat functionele Treg geïnduceerd waren.

In hoofdstuk 6 werd Mt Hsp70 nasaal toegediend en werd gevonden dat Mt Hsp70 ook via deze route PGIA kan onderdrukken. Voor humane toepassing zou behandeling met Hsp70-peptiden, in plaats van het hele eiwit, het voordeel kunnen hebben dat het specifieker kan aangrijpen. Om te na te gaan welke peptiden van Mt Hsp70 betrokken zijn bij bescherming is een epitoop mapping gedaan. Hierbij werd gevonden dat het sterk geconserveerde C1 peptide een dominant T cel epitoop is en bovendien voor de productie van het regulatoire cytokine IL-10 zorgt. Daarnaast vonden wij dat T cellen specifiek voor het bacteriële C1 peptide, ook de muis homologen mC1a en mC1b konden herkennen. Nasale behandeling met C1, mC1a of mC1b kon vervolgens het ontwikkelen van artritis onderdrukken daarmee aantonend dat de peptiden ook immuunregulatie kunnen induceren. In een aantal eerdere studies is al aangetoond dat peptiden uit de C1 regio op het MHC van zowel muis als mens gevonden kunnen worden. Doormiddel van een computer algoritme konden wij laten zien dat C1 peptiden inderdaad aan het humane MHC kunnen binden. Daarnaast vonden wij dat T cellen van gezonde controles maar ook T cellen geïsoleerd uit het ontstoken gewricht van een JIA en een RA patiënt (m)C1 peptiden kunnen herkennen. Dit laat zien dat ook het humane immuunsysteem deze peptide kan herkennen.

Tot slot is in hoofdstuk 7 het effect van manipulatie van de Hsp70 expressie zelf op ontstekingsziekte bestudeerd. Om Hsp70 op te reguleren werd gebruik gemaakt van carvacrol. *In vitro* bleek dat carvacrol een zeer potente co-inducer van Hsp70 is, zowel muis als humane immuuncellen. Orale toediening van carvacrol aan muizen zorgt ook *in vivo* voor een toename in Hsp70 in de Peyerse platen (lymfoïde structuren op de darm). Daarnaast verhoogde de toediening van carvacrol de Hsp70-specifieke T cel respons. Behandeling met carvacrol, voor induceren van PGIA, kon het ontwikkelen van artritis bijna helemaal voorkomen. Ook T cellen, geïsoleerd uit carvacrol behandelde muizen beschermden tegen het ontwikkelen van artritis wat aantoonde dat de bescherming T cel gemedieerd is. Deze studie laat voor de eerste keer zien dat het immuunsysteem inderdaad kan reageren op veranderde Hsp expressie. Daarnaast laat het zien dat voedsel componenten zoals carvacrol immuunregulatoire mechanismen kunnen bevorderen.

De studies beschreven in dit proefschrift dragen in de eerste plaats bij aan het algemene inzicht in hoe Hsp70 een rol speelt bij het handhaven of herstellen van de balans tussen pro- en anti-inflammatoire reacties. Dit inzicht zal essentieel zijn voor het ontwikkelen of verfijnen van immunotherapie in auto-immuunziekten. Daarnaast illustreren de beschreven studies hoe Hsp gebruikt kunnen worden om op een specifieke manier een verstoorde balans te herstellen. Door het verbeteren van de Hsp-specifieke T cel respons met behulp van het hele Hsp eiwit of Hsp-peptiden of door het verhogen van de stress induceerbaarheid van Hsp70 zelf. De pre-klinische studies beschreven in dit proefschrift kunnen dan ook een belangrijke stap zijn op weg naar immunotherapie met Hsp70-peptiden of co-inducers in de patiënt.

Dankwoord

Dankwoord

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Jacqueline en Michiel jullie vriendschap is mij nog steeds heel dierbaar. De tijd dat we elkaar elke dag zagen is helaas voorbij maar ik hoop dat het, ondanks de afstand, weer wat vaker lukt om af te spreken dan de afgelopen tijd.

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''And every day of my life is filled with loving you...." *(Minnie Ripperton)*
Frans, dank je wel voor wie je bent en voor wat je voor mij doet.

Curriculum Vitae
and
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Curriculum Vitae

Lotte Wieten werd geboren op 9 maart 1976 te Amsterdam. In 1994 behaalde zij het VWO diploma aan de Kees Boeke school te Bilthoven. In datzelfde jaar werd een begin gemaakt met de studie Biologie aan de Universiteit van Utrecht. Tijdens haar studie liep zij stage bij de Immunotoxicologie groep van het Institute of Risk Assessment Sciences (IRAS) te Utrecht. Onder begeleiding van Dr. Raymond Pieters wekte zij daar aan een project getiteld: activatie van nuclear factor κB (NFκB) door laag moleculaire immuunstimulerende stoffen. Tijdens haar tweede stage, bij de afdeling Pathologie van het Universitair Medisch Centrum Utrecht (UMCU), deed zij onderzoek naar de invloed van connective tissue growth factor (CTGF) op extracellulaire matrix productie en wondheling in renale mesangium cellen, onder supervisie van Dr. Ingrid Blom en Dr. Roel Goldschmeding. In 2001 werd het doctoraal diploma behaald met als specialisatie Fundamenteel Biomedische Wetenschappen. Hierna was zij werkzaam als research analist bij de afdeling Pathologie van het UMCU. In de groep van Dr. Roel Goldschmeding deed zij onderzoek naar de rol van CTGF in diabetische nefropathie. Daarnaast werkte zij tijdens deze periode op een internationale dressuurstal waar zij meerdere paarden en ruiters trainde en deelnam aan wedstrijden.
Van maart 2004 tot juni 2008 werkte zij als assistent in opleiding bij de afdeling Infectie ziekten en Immunologie van de faculteit diergeneeskunde te Utrecht. Onder begeleiding van Dr. Ruurd van der Zee, Dr. Femke Broere en Prof. dr. Willem van Eden resulteerde haar promotieonderzoek naar endogene stress eiwitten als doelwit voor anti-inflammatoire T cellen uiteindelijk in dit proefschrift. Tijdens haar promotieperiode nam zij plaats in de onderwijs commissie van de Eijkman Graduate School en was zij lid van de aio commissies van de faculteit diergeneeskunde en de Utrecht Graduate School of Life Sciences (UGS-LS). Als representant van deze laatste was zij ook lid van de daily board of studies van UGS-LS.
Vanaf augustus 2008 is zij werkzaam als post-doctoraal onderzoeker bij Prof. Dr. Marcel Tilanus op de afdeling Transplantatie Immunologie in het Universitair Medisch Centrum Maastricht (MUMC).

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