Cell Stress and Chaperones (2010) 15:25–37
DOI 10.1007/s12192-009-0119-8

ORIGINAL PAPER

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

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Received: 17 March 2009 / Revised: 26 March 2009 / Accepted: 19 April 2009 / Published online: 27 May 2009

Abstract Stress proteins such as heat shock proteins (Hsps) are up-regulated in cells in response to various forms of stress, like thermal and oxidative stress and inflammation. Hsps 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 readout systems to monitor Hsp70 expression in a 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, among 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.

Keywords Hsp · Food components · T-cell · Immune modulation · Analysis

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Introduction

Heat shock proteins (Hsps) are intracellular proteins important for maintenance of cellular and immune homeostasis (Lindquist and Craig 1988). Through their chaperone and refolding capacity, Hsps assist in transport and refolding of damaged proteins, processes that are essential to maintain cellular integrity during stress (Fink 1999; Hartl 1996). Hsps are up-regulated in response to various forms of stress, like oxidative, heat, and inflammatory stress (Hartl et al. 1992). Based on their molecular weight, Hsps 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 (Kampinga et al. 2009;

Daugaard et al. 2007). Transcription of inducible Hsp is initiated by binding of heat shock factor 1 (HSF1) to heat shock binding elements (HSE) in the promoter region (Morimoto 1993). 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 (Schett et al. 1998; De Graeff-Meeder et al. 1991; Boog et al. 1992). The immunoregulatory potential of stress proteins is becoming increasingly clear (Cohen 2007; van Eden et al. 1988, 2007). 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 van Eden et al. (2005)). Also in recent clinical trials in patients with inflammatory disease, administration of Hsp-derived peptides has been proven successful (Raz et al. 2001; Prakken et al. 2004).

Decreasing stress inducibility of Hsp with increasing age has been reported (Visala Rao et al. 2003; Njemini et al. 2002). Furthermore, altered Hsp expression has been associated with immune dysfunctions (Miyata et al. 1999; Burkart et al. 2008). 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 (Zou et al. 1998; Morimoto and Santoro 1998; Soti and Csermely 2006). 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 (Katsuno et al. 2005; Westerheide and Morimoto 2005; Vigh et al. 1997; Smith et al. 1998; Brundel et al. 2006). 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 the three techniques, we detected novel compounds that enhance expression of Hsps.

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 Hsps 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 naive BALB/c mice were exposed to a 1-h heat shock (HS) at 42.5°C. After a 3-h recovery at 37°C, Hsp60 and Hsp70 protein expression were determined by western blot analysis. In spleen cells incubated at 37°C, Hsp70 expression was very low; a HS treatment resulted in a strong increase (Fig. 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 6 h at fever range temperature (39°C) as described previously (Pritchard et al. 2004); subsequently, mice were kept overnight at room temperature. WBH enhanced core body temperature of the mice up to 39°C (±0.5; Fig. 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 (Fig. 1c). In agreement with our in vitro results, no effect on Hsp60 expression was detected. Since in vitro heat shock has been reported to induce Hsp expression with different kinetics and optimal temperatures in T- and B-cells (Gothard et al. 2003), 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 h after in vivo WBH and measured surface expression of CD3, CD19, and MHC class II by flow cytometry. WBH treatment did not obviously influence the percentages of CD3, CD19, or MHC class II positive cells (Fig. 1d), illustrating that the effects on Hsp expression

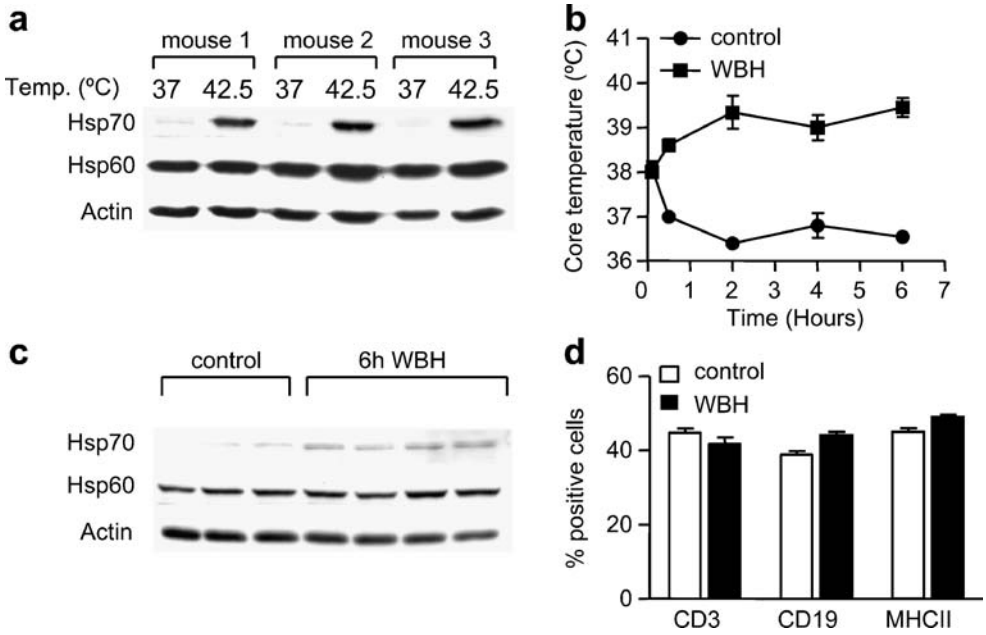

Fig. 1 Hsp60 and Hsp70 are differentially induced in immune cells by raised temperature. **a** Primary spleen cells were isolated and heat-shocked in a waterbath at 42.5°C, control cells were kept at 37°C. After 3 h of recovery at 37°C, Hsp60 and Hsp70 expression was measured by western blot analysis. Actin was used as loading control. **b–d** Mice were whole body hyperthermia (WBH)-treated for 6 h in an incubator at fever range temperatures (39°C), whereas control mice were kept at room temperature. **b** Core body temperature was measured by implanted transponders and expressed as mean (±SD) of two mice. Data are representative of two independent experiments. **c** Hsp60 and Hsp70 protein levels were measured by western blot analysis of cell lysates obtained from spleen cells of mice after 18 h of recovery at room temperature. Data show three (control) and four (WBH) mice. **d** Eighteen hours after WBH, spleen cells were isolated and surface marker expressions of CD3, CD19, and MHC class II were determined by flow cytometry. Data are expressed as mean (±SEM) percentage positive cells of total spleen cells obtained from $n=3$ (control) or $n=4$ (WBH) mice per group

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 in spleen cells.

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 (Yan et al. 2004; Brundel et al. 2006; Kato et al. 1998), 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 (Bachelet et al. 1998). 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 1 h HS at 42.5°C, cells were allowed to recover for 18 h 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 (Fig. 2a). In addition, analysis of the kinetics of Hsp70 expression in these cell types revealed that HS induced Hsp70 levels peaked between 4 h and overnight recovery (Fig. 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 (Kato et al. 1998; Zou et al. 1998). 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 2 h with one of the compounds followed by 1 h HS at 42.5°C or only exposed to the HS. After 18 h 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 dimethyl sulfoxide (DMSO; Fig. 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 geldanamcin slightly augmented the expression of Hsp70 (data not shown). Similar results were

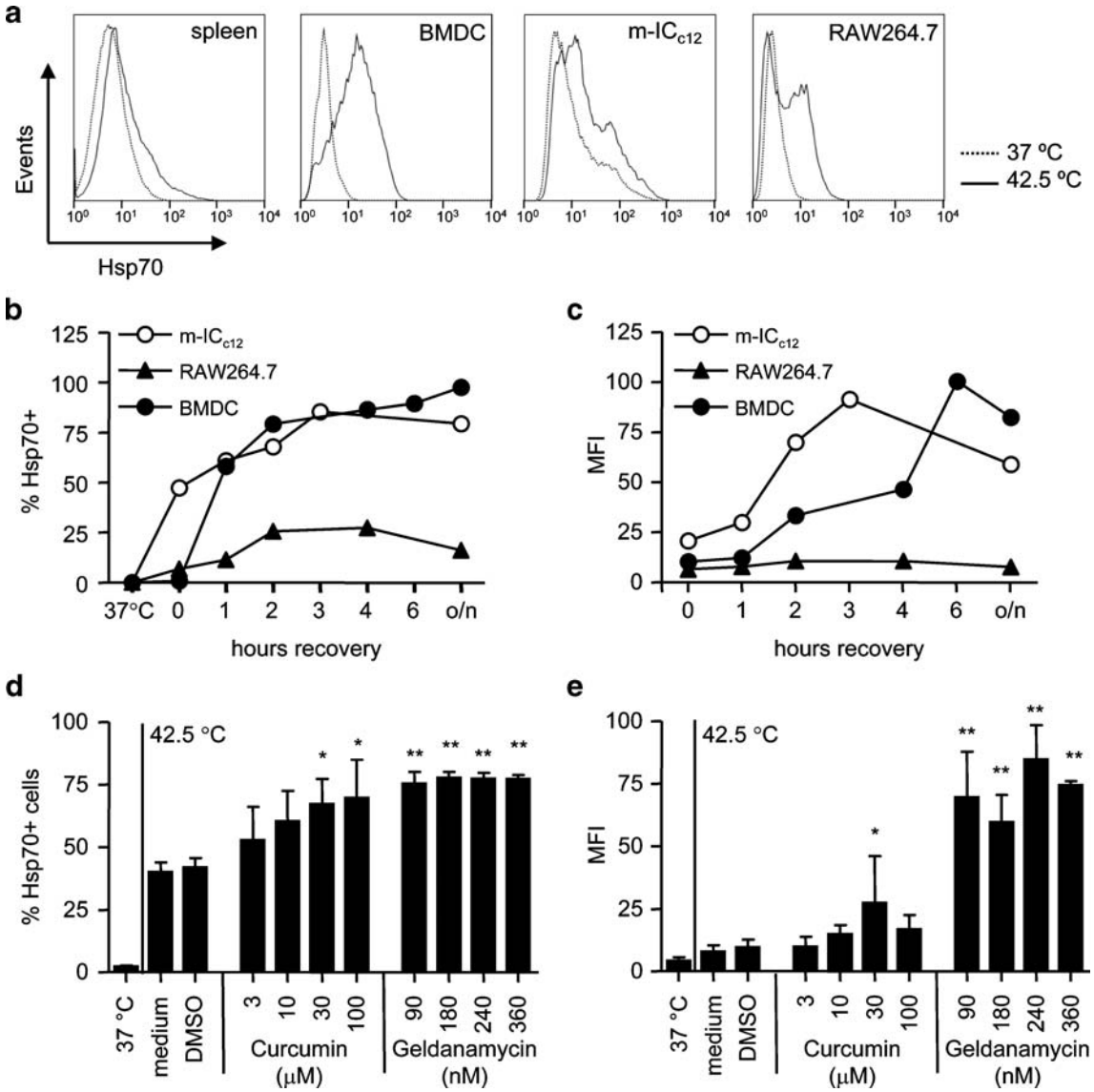

Fig. 2 Flow cytometry allows efficient detection of manipulated Hsp70 expression. **a–c** RAW264.7 cells, m-IC$_{c12}$ intestinal cells, primary spleen, and bone marrow-derived dendritic cells (BMDC) were heat shock-treated for 1 h at 42.5°C or kept at 37°C. After recovery at 37°C for an indicated time, 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 of at least three independent experiments. **b**, **c** Kinetics of Hsp70 induction; **b** % Hsp70$^+$ cells of total viable cells. **c** Mean fluorescence intensity (*MFI*) of Hsp70$^+$ cells. **d**, **e** RAW264.7 cells were either or not incubated at 37°C for 2 h with curcumin, geldanamycin, or the vehicle DMSO (at 0.2%) followed by 1 h heat shock at 42.5°C and overnight recovery at 37°C. Graphs show (**d**) % Hsp70$^+$ cells of total viable cells and (**e**) MFI of total viable cells. Data are depicted as means (±SEM) of three independent cultures. Statistical differences *$p<0.05$ and **$p<0.01$ were calculated by ANOVA (two tailed) with Bonferroni correction for multiple comparisons

obtained in epithelial cells and BMDC (data not shown). In summary, this demonstrated that flow cytometry allowed efficient detection of compound enhanced Hsp70 expression.

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 (Pellegrini et al. 2003) and of carvacrol, the major constituent of essential oils obtained from the herbs oregano and thyme (Skoula et al. 1999). For five food extracts, both a lipophilic and a hydrophilic fraction were 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 (Fig. 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

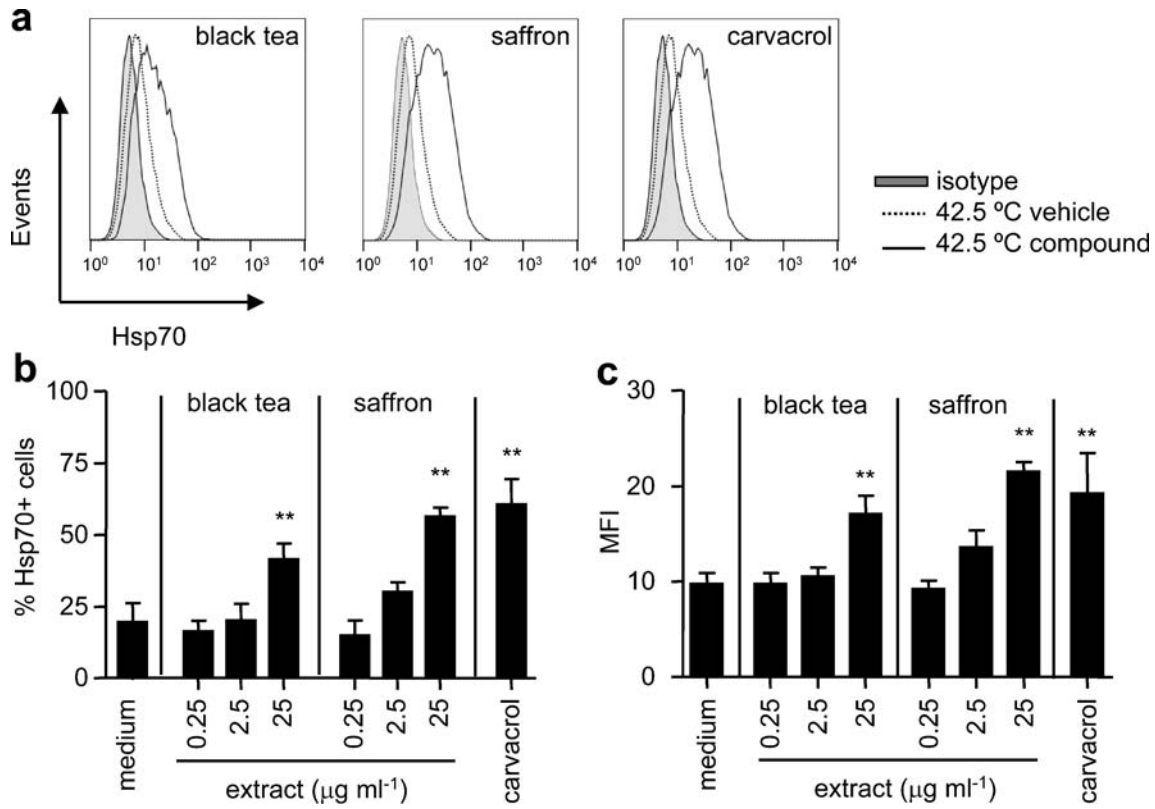

Fig. 3 Extracts of black tea and saffron and carvacrol co-induce Hsp70 expression. Incubation of RAW264.7 cells with hydrophilic fractions of black tea and saffron extracts or 0.2 mM carvacrol was done for 2 h at 37°C, followed by 1 h heat treatment at 42.5°C. After overnight recovery at 37°C, intracellular Hsp70 was measured by flow cytometry. **a** Histograms show Hsp70 expression in viable cells. **b**, **c** Graphs show **b** % Hsp70$^+$ cells and **c** mean fluorescence intensity (*MFI*) of total viable cells. Data in **b** and **c** show means (±SEM) of triplicate cultures and are representative of two independent experiments. Statistical differences $^{**}p<0.01$ were calculated by ANOVA (two tailed) with Bonferroni correction for multiple comparisons

(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 (Fig. 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.

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 h with the mC1b peptide and irradiated A20 lymphoma B cells as APC. 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 Fig. 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 (Fig. 4b). For some T-cell hybridomas, activation-induced cell death has been reported (Ashwell et al. 1987). 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}annexin V^{neg} cells, while the percentage of non-viable 7AAD^{pos}annexin V^{pos} cells was increased (Fig. 4c). Both IL-2 production and AICD could be specifically blocked by blocking antibody to MHC class II (data not shown). Then, we studied whether the

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
Blueberry	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) and 25 μg ml^{-1} (all other extracts) for 2 h 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 1 h 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

hybridomas can recognize the endogenous Hsp70. We exposed APC to 1 h HS and let them recover for 3 h 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 (Fig. 5). As a specificity control, we used the 5/4E8 hybridoma (Brennan et al. 1995) 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 shown). Thus, the CD4$^+$ hybridoma T-cell clones we isolated differ in recognition of endogenous Hsp, even though they all recognize the mC1b peptide.

Hsp70-specific CD4$^+$ 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 2 h followed by 1-h HS at 42.5°C and 3-h 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 (Fig. 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.

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 messenger RNA (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 (Fig. 7). For carvacrol to act, at least some stress is needed: no effect is seen at 0 and 2.5 mM arsenite while at 5 mM 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.

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 (Ellis 1990; Lindquist and Craig 1988; Morimoto 1993). Boosting the heat shock response with food-derived compounds may be an attractive way to modulate Hsp expression. Here, we

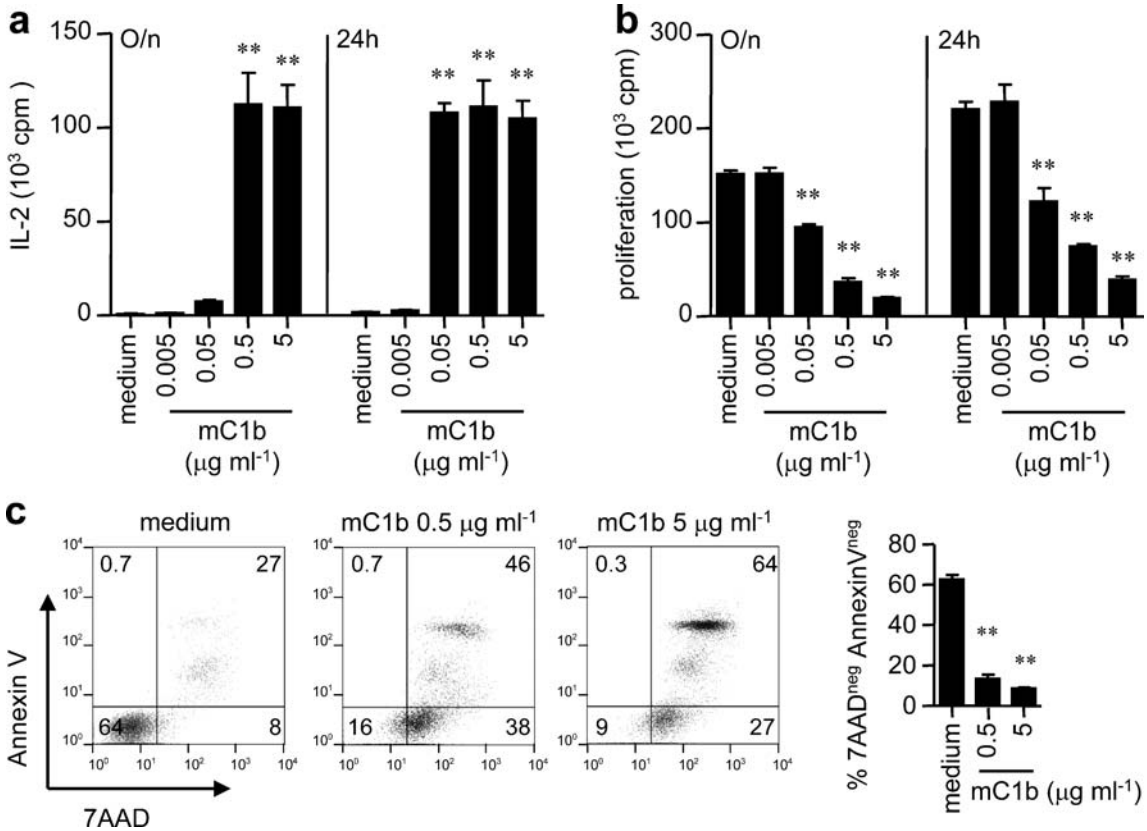

Fig. 4 Hsp70-specific CD4$^+$ T-cell hybridomas show enhanced IL-2 production and reduced proliferation upon activation. Hybridomas specific for the immunodominant Hsp70 mC1b peptide (aa 169–183) were generated by immunization of BALB/c mice with the peptide. Subsequently, fusion of spleen cells isolated from these mice with BW5147 cells was followed by limited dilution cloning of CD4$^+$ T-cell hybridomas. **a–c** LHEP7, as representative example of obtained clones, was incubated for 24 h with antigen-presenting cells (A20). To some cultures, mC1b peptide at indicated concentrations was added. **a** Bioactive 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** and **b** are expressed as mean 10^3 cpm (±SEM) 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 $^{**}p<0.01$ and were calculated by ANOVA (two tailed) with Bonferroni correction for multiple comparisons

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 T-cell hybridomas. These hybridomas specifically recognize an immunodominant mouse Hsp70 peptide in a MHC class II-dependent fashion and respond to enhanced Hsp70 levels in antigen-presenting cells. Third, we generated a luciferase reporter system to investigate if compounds sensitize cells to stress or enhance the stress response.

In many previous studies, analysis of Hsp70 expression has been performed by western blot (Brundel et al. 2006; Kato et al. 1998; Kieran et al. 2004; Yan et al. 2004). 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 in agreement with previous literature found that Hsp70 expression was increased after pre-incubation of RAW264.7 cells with geldanamycin (Zou et al. 1998) or curcumin (Kato et al. 1998) 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

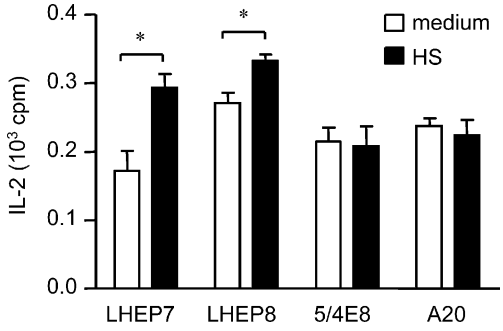

Fig. 5 Hsp70-specific CD4$^+$ T-cell hybridomas can recognize endogenous Hsp70. A20 cells were incubated at 42.5°C for 1 h (HS), or kept at 37°C (medium). After 3 h of recovery at 37°C, A20 cells were used as antigen-presenting cell to stimulate LHEP7 and LHEP8. As a specificity control, A20 cells were incubated with the 5/4E8 T-cell hybridoma specifically recognizing a proteoglycan-derived peptide (aa 70–84). After 24 h culture supernatants were harvested and IL-2 production by LHEP7, LHEP8, 5/4E8, or A20 cells alone was determined by incubation with the IL-2-dependent CTLL-16 cell line. Data are expressed as mean 10^3 cpm (±SEM) of triplicate cultures and are representative of two independent experiments. Significant differences *$p<0.05$ were calculated by ANOVA (two tailed) with Bonferroni correction for multiple comparisons

increased presentation of Hsp70 peptides on MHC class II. Also, Hsp can act as the 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-cell subsets recognize ergotopes in an MHC-restricted manner but also MHC-independent induction of gamma-delta T-cells with regulatory capacity has been described (Cohen et al. 2004; Quintana and Cohen 2006). 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 these data, our previous study in rats showed that Hsp60-specific T-cells recognized up-regulation of cellular Hsp60 in the APC (Paul et al. 2000). The mC1b peptide from mouse HspA1A has been frequently eluted from MHC class II (Halder et al. 1997; Chicz et al. 1993; Dengjel et al. 2005). 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 readout 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 (Hattori et al. 1993; Kampinga et al. 2009; Qiu et al. 2006). 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 favorable disease outcome in patients with juvenile idiopathic arthritis or rheumatoid arthritis (de Graeff-Meeder et al. 1995; Kamphuis et al. 2005). 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 (Massa et al. 2007). 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 (Nishikawa et al. 2005). Also, enhanced expression of DNAJ family members in the synovial tissue of RA but OA patients has been found (Kurzik-Dumke et al. 1999). These studies underlined that compounds acting on DNAJ and

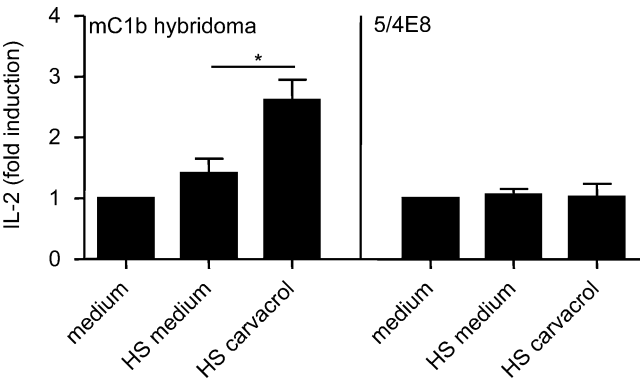

Fig. 6 Hsp70-specific T-cell hybridomas can respond to compound induced up-regulation of Hsp70. Antigen-presenting cells (A20) were incubated or not with 0.2 mM carvacrol for 2 h followed by 1 h heat shock at 42.5°C. Control cells were kept at 37°C. After 3 h of recovery, A20 cells were cultured overnight or for 24 h with mC1b-specific hybridomas. As specificity, control A20 cells were cultured for 24 h with the 5/4E8 hybridoma. IL-2 production, upon hybridoma activation, in collected supernatants was determined as increased [^{3}H]-incorporation by the IL-2-dependent CTLL-16. Data are expressed as mean induction (±SEM) relative to medium non-HS treatment (cpm medium/cpm HS or HS + carvacrol). mC1b data show pooled data of LHEP7, LHEP13, LHEP19, and LHEP24. Significant differences as compared to medium control 37°C APC are indicated by *$p<0.05$. All statistical differences were calculated by ANOVA (two tailed) with Bonferroni correction for multiple comparisons

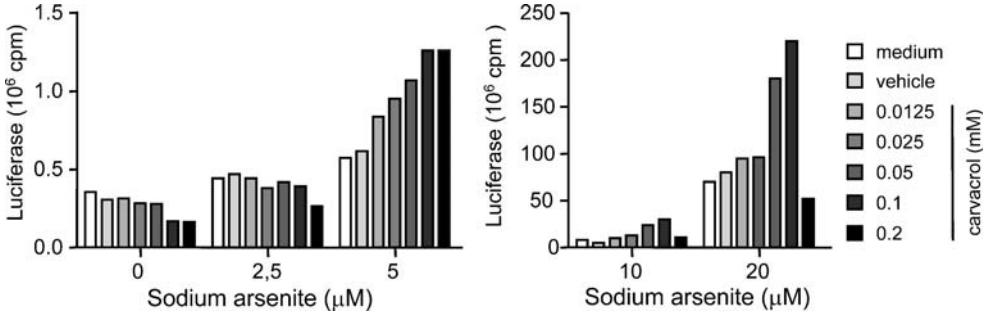

Fig. 7 Analysis of HSF1 activation with DNAJB1-luc-O23 reporter cells. Reporter cell lines stably expressing a fragment containing the sequence from −500 to +41 of the human DNAJB1 (Hsp40) gene were generated as described in the "Materials and methods". Cells were incubated with carvacrol and sodium 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

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 (Verbeke et al. 2001; Rao et al. 1999). 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.

Materials and methods

Cell culture

RAW264.7 and A20 cell lines were purchased from American Type Culture Collection. CTLL-16 (Gillis et al. 1978) was obtained from Sanquin Blood Bank. A20 and CTLL-16 were routinely cultured in Optimem supplemented with 5% fetal bovine serum (FBS; Bodinco B.V.). RAW264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% FBS and 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (p/s; Gibco BRL). m-IC_{c12} intestinal cells, primary spleen cells, and BMDC were cultured in IMDM with 10% FBS and p/s. For spleen and BMDC cultures, 5×10^{-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 (Lutz et al. 1999) 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 (Pellegrini et al. 2003, 2006). In brief, hydrophilic fractions of samples from fruit and vegetables were obtained by extracting a homogenized sample with water under agitation for 15 min at room temperature. The supernatant was collected after 10 min of centrifugation at 1,000×*g*. 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 1,000×*g*. 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 1,000×*g*, the supernatant was collected.

In vitro heat treatment and exposure to compounds

For in vitro heat shock treatment, cells were seeded in 24- or 48-well plates at 1–2 ml per well (2×10^6 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 2 h 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 2 h, the plates were sealed and placed for 1 h in a waterbath preheated at exactly 42.5°C. After that, cells were allowed to recover at 37°C for an 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. WBH treatment was done as described previously (Pritchard et al. 2004). In brief, 7 days before the start of the WBH treatment, temperature-sensitive transponders (IPTT-300; Plexx, Elst, The Netherlands) were implanted subcutaneously in the dorsal thoracic skin. At the start of WBH treatment, mice were placed in preheated cages and transferred into a preheated incubator. During the experiment, the 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 a wireless transponder readout (WRS-6006/6007; Plexx). Control mice were handled in the same way but at room temperature. After 6 h, mice were transferred to cages at room temperature. After 18 h, mice were sacrificed 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 phosphate-buffered saline (PBS). Subsequently, pellets were resuspended in 2× 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 1× Laemlli sample buffer to a final concentration of 1.5 mg ml^{-1}. After 5 min of 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}; Cornelussen et al. 1997; 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). 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 four 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 Biosciences Europe GmbH).

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 min with Cytofix/Cytoperm solution (BD Pharmingen), washed, and then incubated with either antibody to Hsp70 fluorescein isothiocyanate labeled (SPA810; Stressgen), specifically recognizing the 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 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. After 48 h, viable cells were isolated using LympholyteM (Cedarlane Laboratories) and cultured for another 48 h in conditioned medium (IMDM supplemented with 10% FBS, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 5×10^{-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 (Kappler et al. 1981). By FACS Vantage (BD), fused cells were seeded into 96-well plates at one cell per well. Analysis of mC1b specificity of the obtained clones was performed by incubation of hybridoma cells (2×10^4 per well) with APC (A20 B lymphoma cells, 2×10^4 per well) in 96-well flat bottom plates for 24 h with or without peptides at indicated concentrations. Additionally, A20 cells were either or not heat-shocked (1 h in a water bath at 42.5°C) followed by 2–3 h recovery at 37°C and were afterwards used as APC. Incubation with carvacrol was

done for 2 h at 0.2 mM prior to heat shock treatment. After overnight, 24 or 48 h of culturing, harvested supernatants were cultured with IL-2 responder CTLL-16 cells for 24 h, pulsed overnight with [^{3}H]-thymidine (0.4 μCi per well; Amersham Biosciences Europe GmbH), and 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 (Brennan et al. 1995) 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 aagtcgaccagacacaggttagg-tagttcgtcc and accatggcccctcctgcggcccgccga and cloned *Sal*I/*Nco*I in the *Xho*I and *Nco*I sites of pGL3basic (Promega). The *Nhe*I/*Bam*HI 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 5×10^4 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 h 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 the 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.

Acknowledgments We thank Jolanda van den Brink, Josée Wagenaar-Hilbers, and Peter van Kooten for technical support and Prof. Dr. Luc Snoeckx for providing the Hsp70 antibody. This work was supported by grants of SenterNovem IOP Genomics project nr IGE03018 and IGE07004, the European Union FP7 TOLERAGE: HEALTH-F4-2008-202156, and TI Pharma Project D5-106.

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