CHARLES UNIVERSITY IN PRAGUE

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Dissertation thesis

# The role of heat shock proteins in pathogenesis of leukaemia, graft *versus* host disease, juvenile idiopathic and rheumatoid arthritis

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# 1 The aims of the study

Heat shock proteins (Hsps) represent major immunodominant antigens in a wide spectrum of microbial pathogens and immune reactivity against different members of HSP families accompanies many infectious diseases. Known role of Hsps in antigen presentation could also suggest their potential role in the alloreactive process that leads to acute graft *versus* host disease (GvHD). Sequence homology and cross-reactivity between microbial and human Hsps led to the concept that Hsps might be involved in the etiopathogenesis of autoimmune diseases such as juvenile idiopathic arthritis (JIA) and rheumatoid arthritis (RA). The major groups of molecular chaperones have been implicated in cancer development as well and stress-inducible Hsp70 was found to be present on the plasma membrane of tumour cells but not on the corresponding normal tissues.

On the basis of previous observations we focused our study on the role of Hsps, namely Hsp60 and Hsp70, in the pathogenesis of leukaemia, graft *versus* host disease, juvenile idiopathic arthritis and rheumatoid arthritis.

## 1.1. Investigation of peripheral blood mononuclear cell responses to heat shock proteins and their derived synthetic peptides in patients undergoing stem cell transplantation and in patients suffering from juvenile idiopathic arthritis

We studied peripheral blood mononuclear cell (PBMC) responses to recombinant human Hsp60 (rh-Hsp60), *Mycobacterium bovis* Hsp65 (*M. bovis* Hsp65) and recombinant human Hsp70 (rh-Hsp70) in relation to acute GvHD and infection in paediatric patients with various lympho-hemopoietic malignancies as well as non-malignant disorders subjected to stem cell transplantation (SCT). JIA patients were screened for PBMC responses to rh-Hsp60, *M. bovis* Hsp65 and rh-Hsp70 and their derived peptides. Cell responses were measured after the incorporation of $^{3}$H-thymidin and were expressed as stimulation indexes (SI).

## 1.2. Analysis of cell surface and relative gene expression of Hsp70 in leukaemia and RA patients

Stress-inducible Hsp70 cell surface and mRNA expression was studied in human leukaemia cell lines (K562, Jurkat, CCRF-CEM) during several passages of in vitro culture by using flow cytometry and real-time quantitative reverse-transcriptase polymerase chain reaction (RT-PCR).

Next goal of the study was to determine cell surface and mRNA expression of stress-inducible Hsp70 in synovial cells derived from synovial tissue of RA patients who underwent synovectomy. Simultaneously, Hsp70 expression was studied in autologous skin dermis gained simultaneously from operation wound and peripheral blood leukocytes of RA patients and healthy controls.

## 2 Methods

Overall 7 methods were used in the studies related to the topic of the dissertation thesis. The methods are described in details in the publications.

1. Cell proliferation assay with $^{3}$H-thymidine incorporation
2. Mixed lymphocyte culture assay
3. Solid phase method of peptide synthesis
4. Cell cultivation and preparation
5. Flow cytometry
6. RNA isolation
7. Real-time quantitative RT-PCR

# 3 Results and discussion

The results of the presented dissertation study are formed by findings of four papers which were accepted for publication in international journals.

## 3.1. Results to aim 1

### 3.1.1. Peripheral blood mononuclear cell responses to heat shock proteins in patients undergoing stem cell transplantation

(published in *Sedlackova L, Sedlacek P, Hromadnikova I. Peripheral blood mononuclear cell responses to heat shock proteins in patients undergoing stem cell transplantation. Pediatr Transplantation 2006;10:178-186.*)

We studied proliferative responses of PBMC to Hsps (rh-Hsp60, *M. bovis* Hsp65 and rh-Hsp70) in relation to anamnestic or actual bacterial and/or fungal infection and GvHD in 34 patients with malignant and non-malignant diseases indicated to SCT. 37 healthy controls were also included in this study. Cell proliferation was measured by $^{3}$H-thymidine incorporation. The most common method is to calculate SI, which is the stimulated cell mean cpm divided by the mean cpm of unstimulated cells. Phytohemagglutinin (PHA) was used as a control of the stimulation ability.

#### *3.1.1.1. Proliferative responses to Hsps in relation to anamnestic or actual infection*

Only a cohort of patients before SCT with responsiveness to PHA (SI > 40) was chosen for the analysis to exclude immunocompromised patients not yet recovered from the previous chemotherapy.

PBMC from patients with anamnestic or actual infection showed higher proliferative response to stimulation with rh-Hsp60 and *M. bovis* Hsp65 when compared with patients without infection ($P = 0.004$, $P = 0.007$) or healthy controls ($P = 0.01$, $P = 0.01$), although responses to PHA were not significantly different between the two groups ($P = 0.57$, $P =$

0.71). However, proliferation responses to rh-Hsp70 did not reach statistical significance between these two cohorts (P = 0.20, P = 0.88). When we compared proliferative response with PHA (P = 0.28, 0.91), rh-Hsp60 (P = 0.04, 0.02), *M. bovis* Hsp65 (P = 0.03, 0.02) and rh-Hsp70 (P = 0.25, 0.78) between an infectious group and healthy children and/or healthy adults only, no statistical difference was found. Strong PBMC responses to rh-Hsp60 and/or *M. bovis* Hsp65 were found in patients who were at present colonized with *Escherichia coli* (n = 1) and *Klebsiella pneumoniae* (n = 1) or had previously *K. pneumoniae* infection with subsequent sepsis (n = 2). In addition, there was a significantly lower proliferative response to rh-Hsp60 (P = 0.01), *M. bovis* Hsp65 (P = 0.006) and rh-Hsp70 (P = 0.02) from PBMC cells obtained from a cohort of paediatric patients without anamnestic and/or actual infection when compared with healthy adults. Interestingly, the response to PHA did not differ significantly between these two groups (P = 0.16). When we compare proliferative response with PHA (P = 0.45), rh-Hsp60 (P = 0.19), *M. bovis* Hsp65 (P = 0.57) and rh-Hsp70 (P = 0.92) between a non-infectious group and healthy children only, no statistical difference was found.

Members of HSP families were shown to be the major antigens of many pathogenic organisms and they are known to induce very strong humoral and cellular immune responses in numerous infections (Zügel and Kaufmann, 1999). Different Hsp cognate proteins have a high degree of sequence homology among various pathogenic or non-pathogenic bacteria (Shinnick, 1991), for example, Hsp60 in mycobacteria is homologous to the common antigen of *Pseudomonas aeruginosa* and to Hsp60 of other gram-negative bacteria, including GroEL of *E. coli* (Shinnick et al., 1988; Young et al., 1988). Our findings support various studies dealing with immunodominant Hsps in connection with selected pathogens. Cellular and/or humoral immune response to 60 kDa Hsps was described in *K. pneumoniae* (Dominguez-Lopez et al., 2000; Cancino-Diaz et al., 1998), *E. coli* (Lamb et al., 1989) and *Pseudomonas aeruginosa* (Shinnick et al., 1988; Young et al., 1988) infected patients. The high response in patients with an ongoing infection may be because of the synergistic effect between in vivo stimulation and in vitro stimulation with Hsps.

#### *3.1.1.2. Proliferative responses to Hsps in relation to acute GvHD*

Initially, PBMC responses against rh-Hsp60, *M. bovis* Hsp65 and rh-Hsp70 were compared in a total cohort of patients with acute GvHD onset involving as well as those heavily immunocompromised unable to respond to PHA stimulation (SI = 1–326, median 6)

and patients with no sign of GvHD (SI = 12.2–340, median 46) during equal posttransplant period (11–96 days, median 29.5). Significantly lower responses for rh-Hsp60 (P = 0.005), *M. bovis* Hsp65 (P = 0.004) and rh-Hsp70 (P = 0.04) as well as for PHA (P = 0.008) were detected in a cohort of patients with acute GvHD onset. When only a cohort of GvHD patients with responsiveness to PHA (SI > 5) was chosen for the statistical analysis to evaluate only responders, no statistical difference for proliferative response to PHA was observed (P = 0.37) between these two groups, while proliferative responses to rh-Hsp60 (P = 0.003), *M. bovis* Hsp65 (P = 0.01) and rh-Hsp70 (P = 0.05) remained significantly lower in a group of patients with acute GvHD onset.

Furthermore, responses to Hsps in both cohorts of patients with acute GvHD onset and patients with GvHD later re-activation were tested. Comparing these two cohorts no significant difference in responses to rh-Hsp60 (P = 0.36), *M. bovis* Hsp65 (P = 0.25) and rh-Hsp70 (P = 0.21) was found. As expected, SI for PHA reached higher values in patients with GvHD later re-activation (1.3–229, median 50) as distinct from patients with acute GvHD (1–326, median 6), however, this difference did not reach statistical significance (P = 0.07). Similarly, when only patients with responsiveness to PHA (SI > 5) were chosen for the statistical analysis from both GvHD cohorts, no statistical difference for proliferative response to rh-Hsp60 (P = 0.10), *M. bovis* Hsp65 (P = 0.14) and rh-Hsp70 (P = 0.86) between these two groups was found.

In this study, when we included only patients with myeloablative preparative regimen (Busulphan/Cyclophosphamide, total body irradiation/Etoposide, etc.), we got the same statistical results for both cohorts of patients with acute GvHD onset and GvHD re-activation as mentioned above.

Several lines of evidence propose that Hsps play a role in the alloreactive process that leads to acute GvHD after transplantation. First, Hsps were described to play a role in MHC-antigen processing (Melnick and Argon, 1995). They have been demonstrated to form Hsp-peptide complexes that can initiate antigen-specific cytotoxic T-cell responses (Arnold et al., 1995). These Hsp-chaperoned peptides can be taken up by antigen presenting cells (APCs) before being represented on MHC class I molecules (Suto and Srivastava, 1995). In addition, Hsp70 may enhance the presentation of antigenic determinants including the minor histocompatibility antigens, and promote acute GvHD. Furthermore, Jarvis et al. (Jarvis et al., 2003) found increased expression of inducible Hsp70 in the skin explant biopsy specimens significantly associated with clinical acute GvHD grades II–IV irrespective of GvHD prophylaxis. They suggested that this dramatic induction of Hsp70 in graft *versus* host

reaction might be a result of a protective response in an attempt to refold damaged polypeptides which is correlated with Hsps's known function of protein folding and repair.

We observed predictable values of SI for PHA after SCT, which correlated with clinical findings, found to be higher in patients with less intensive depression of immunity (type of conditioning, lower dose of drugs to prevent GVHD, etc.) (D'Andrea et al.,1986). We presume that lower Hsps stimulations in acute GvHD patients in comparison to patients with no sign of GvHD can be explained neither by immunosuppression induced by GvHD treatment nor by delayed reconstitution of immunity from grafts. However, it may be explained by Van Eden et al. (Van Eden et al., 1998) hypothesis how Hsps could control the balance of T-cell regulation.

Peripheral T-cell responses to self-Hsp are abundant as self-Hsp specific T cells escape from negative thymic selection and low-level expression of self-Hsp epitopes on non-professional APCs leads to the induction of peripheral tolerance. Inflammatory processes, including those resulting from acute GvHD, will lead to stress-induced upregulation of Hsp synthesis at the site of inflammation, which was also confirmed by Jarvis et al. (Jarvis et al., 2003). The upregulated Hsps will attract the peripherally tolerized T cells to the site of inflammation, where the self-Hsp epitopes will trigger a regulatory or suppressive T-cell response via IL-4, IL-10, TGF-$\beta$ cytokine production (Van Eden et al., 1998). Such a phenomenon was described in children with JIA – $T_H1$-type response mediated systemic inflammatory disease. T-cell proliferative responses to Hsp60 were present exclusively in patients with spontaneously remitting oligoarticular forms of the disease but not in patients with progressive (polyarticular or systemic) forms of the disease (De Graeff-Meeder et al., 1995; Prakken et al., 1996). Such responses showed a pattern of fluctuation that suggested that they coincided with the development of remission, i.e. with disease suppression. Analysis of T cells in these patients revealed the production of IL-4 and TGF-$\beta$, and an overexpression of CD30 upon activation with Hsp60, indicating a $T_H2$-type response. Reviewing the pathophysiology of acute GvHD, donor T cells become activated by host alloantigens, release $T_H1$ cytokines, IL-2 and IFN-$\gamma$, which activates macrophages and NK cells. Activated macrophages and NK cells can then be triggered by gut bacteria and by infections to release large quantities of inflammatory cytokines and active nitrogen intermediates, a "cytokine storm", mediating tissue injury. Cell damage from preparative regimens cause as well as transient release of inflammatory cytokines, such as IL-1 and TNF-$\alpha$, that increases the immunogenicity of host APCs (Hakim and Mackall, 1997).

Similarly, we hypothesize that lower Hsps stimulations in a cohort of patients with acute GvHD onset and GvHD later re-activation when comparing with a cohort of patients without

GvHD might indicate the predominance of host-reactive donor $T_H1$ cell population and the lack and/or insufficient $T_H2$ and $T_H3$–type regulatory response resulting in local host tissue damage in the gut, liver and skin. In conclusion, we hypothesize that increased Hsp-specific stimulation may reflect the presence of protecting regulatory T cells preventing the development of $T_H1$-mediated diseases, involving acute GvHD.

#### *3.1.1.3. Proliferative responses in cultures after antigenic and allogeneic stimulation*

In this part of study we determined the proliferative responses to individual antigens and allogeneic stimuli and wished to find whether Hsps (rh-Hsp60, *M. bovis* Hsp65 and rh-Hsp70) are able to stimulate alloreactive lymphocytes. PBMC from HLA mismatched healthy unrelated individuals were cultured with autologous and allogeneic cells with or without PHA and various Hsps. The autologous stimuli induced low levels and the allogeneic stimuli high levels of proliferation (SI = 15.5–56.2) as expected. PHA induced maximal proliferation in both allogeneic (84 922–86 444 cpm) and autologous (79 846–95 333 cpm) arrangements. However, Hsps stimulated allogeneic system more strongly than autologous system. 2.5–24.4 (rh-Hsp60), 5.2– 35.3 (*M. bovis* Hsp65) and 1.8–11.3 (rh-Hsp70) fold cpm values were demonstrated in allogeneic cultures when compared with the cpm from cultures of autologous cells.

In other words, both autologous and allogeneic mixed lymphocyte cultures induced proliferation when appropriate antigen was added to the cell culture, however, significantly higher proliferative response was observed in allogeneic system. This may be explained by the amplification of T-cell and NK-cell activation because of the synergistic effect of HLA class II incompatibility between the unrelated individuals and in vitro Hsp stimulation.

### 3.1.2. Peripheral blood mononuclear cell responses to heat shock proteins and its derived synthetic peptides in juvenile idiopathic arthritis patients

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The aim of our study was to investigate PBMC proliferative responses to Hsps and Hsp-derived synthetic peptides in a cohort of 48 patients with JIA and compare with healthy controls (n = 38). Beside Hsps (rh-Hsp60, *M. bovis* Hsp65 and rh-Hsp70), we tested these peptides: human Hsp60 (562–571) peptide, which shares sequence homology with cytokeratin (545–554), one of the known RA autoantigens (Jones et al., 1993). Next, we also used *M. bovis* Hsp65 (180-188) peptide, whose observed cross-reactivity with cartilage proteoglycan may cause inflammation in the joints and may be involved in the pathogenic effector mechanism (De Kleer et al., 2003). Finally, Hsp70 derived N-terminal-extended 14-mer peptide (TKD, 450–463) was chosen on the basis of described ability to stimulate cytolytic and proliferative activity of NK cells equipotently with full-length Hsp70 protein (Multhoff et al., 2001).

We investigated also an association between the proliferative response to Hsp and Hsp-derived synthetic peptides and clinical characteristics like rheumatoid factor (RF), antinuclear antibodies (ANA), HLA B27, the duration and the activity of the disease. PHA was used as a control of the stimulation ability in assay using the incorporation of $^{3}$H-thymidine.

#### *3.1.2.1. Proliferative responses to Hsps and Hsp-derived synthetic peptides*

While PHA stimulation was not significantly different between the cohort of JIA patients and healthy controls (P = 0.23), PBMC proliferative responses to rh-Hsp60 (6/43 x 3/38), *M. bovis* Hsp65 (9/47 x 3/36), rh-Hsp70 (8/44 x 2/36) and P180–188 derived from *M. bovis* Hsp65 (9/45 x 1/34) were elevated in a cohort of JIA patients. However, only responses to *M. bovis* Hsp65 reached the statistical significance (P = 0.01). There was no proliferative response to P450–463 peptide derived from rh-Hsp70 in JIA patients. These results are concordant with initial studies reported by Graeff-Meeder et al. (Graeff-Meeder et al., 1991) who described increased proliferative responses to rh-Hsp60 and *M. bovis* Hsp65. Our data

confirmed also the results of Danieli et al. (Danieli et al., 1992) who reported elevated responses to P180–188 *M. bovis* Hsp65 derived peptide in patients with JIA.

Furthermore, PBMC responses to Hsps and Hsp-derived synthetic peptides were compared in various JIA subgroups and healthy individuals. A positive response (SI above mean plus 2 SD of healthy controls) to rh-Hsp60 (4/27), *M. bovis* Hsp65 (6/29), rh-Hsp70 (6/27), P562–571 human Hsp60 (6/29) and P180–188 *M. bovis* Hsp65 (7/29) derived peptides was observed predominantly in patients with polyarthritis. However, only responses to rh-Hsp60 ($P = 0.03$), *M. bovis* Hsp65 ($P = 0.01$) and P562–571 human Hsp60 derived peptide ($P = 0.02$) were significantly above those of healthy controls.

P545–554 cytokeratin derived peptide synthesized on the basis of examination of the entire sequence of human Hsp60 for similarities with some known autoantigens (Jones et al., 1993) showed no proliferative responses in JIA patients. However, P562–571 sequence of human Hsp60 showed significant stimulation in polyarthritis in contrast to P545–554 cytokeratin derived peptide even though they share the sequence homology with only one amino acid interchange. We found no PBMC proliferative responses to P450–463 human Hsp70 derived peptide in JIA patients, which was described to stimulate the cytolytic and proliferative activity of NK cells equally to the full-length Hsp70 protein (Multhoff et al., 2001). This observation is consistent with findings of Multhoff et al. (Multhoff et al., 2001) who described the absence of T cell response against the peptide plus low-dose IL-2 in healthy individuals.

#### *3.1.2.2. Influence of clinical characteristics on proliferative responses to Hsps and Hsp-derived synthetic peptides*

The possible influence of clinical characteristics of JIA patients was also analysed. Patients were subdivided into further groups: RF+ vs. RF-, ANA+ vs. ANA-, HLA B27+ vs. HLA B27-, patients in remission (with or without the therapy) vs. patients with active disease and patients with early (0–2 years) and established disease (patients with duration of the disease over 2 years). No relationship was found between the RF, ANA and HLA B27 presence and the Hsp and Hsp-derived synthetic peptide proliferation in a cohort of JIA patients.

Initially, no relation was found between responses both to Hsps and Hsp-derived synthetic peptides and disease activity when we compared patients in remission with those with active disease. However, when we evaluated the data besides depending on the

therapy in the remission group, we found significantly higher proliferative response to rh-Hsp60 (P = 0.03), *M. bovis* Hsp65 (P = 0.03), rh-Hsp70 (P = 0.02) and P180–188 derived from *M. bovis* Hsp65 (P = 0.004) in a cohort of patients without the therapy. Significantly higher proliferative response to P180–188 derived from *M. bovis* Hsp65 (P = 0.007) was also observed in remission group without the therapy when compared with active disease group. Positive proliferative responses to all Hsps and P562– 571 human Hsp60 and P180–188 *M. bovis* Hsp65 derived peptides were observed exclusively in patients with established disease when compared with patients with early disease. However, only proliferative response to P180–188 derived from *M. bovis* Hsp65 reached the statistical significance (P = 0.04).

We have shown no positive proliferative responses to any Hsps and Hsp-derived peptide in a cohort of patients with early onset of polyarthritis, systemic disease and HLA B27 negative oligoarthritis, none of them achieved the remission. These data correspond with those of De Graeff-Meeder et al. (De Graeff-Meeder et al., 1995) and Prakken et al. (Prakken et al., 1996), who reported significant in vitro T-cell responses to human Hsp60 within 4 weeks to 3 months after the onset of HLA B27 negative oligoarticular JIA with favourable outcome associated with immunoregulatory control leading often to remission when the previous positive responses to rh-Hsp60 were lost. At the early stage of arthritis no highly specific responses for polyarthricular and systemic onset forms of JIA were observed (Prakken et al., 1996).

However, it looks like that the situation in patients with later stages of JIA disease is completely different, since we observed positive proliferative responses to all Hsps and P562–571 human Hsp60 and P180–188 *M. bovis* Hsp65 derived peptides exclusively in patients with established disease. These data can be supported by De Kleer et al. (De Kleer et al., 2003) who found elevated response towards Hsp60 in polyarticular and systemic disease and by Prakken et al. (Prakken et al., 2002) who described responses towards self-Hsp60 in polyarticular JIA at later stages of disease activity.

It was suggested that human Hsp60-specific T cells have regulatory function and contribute to disease remission in oligoarticular JIA patients (De Kleer et al., 2003). De Kleer et al. (De Kleer et al., 2003) also speculate that T cell responses against human Hsp60 in polyarticular JIA patients are qualitatively different; they appear later in the course of the disease, probably through bystander activation, and show no regulatory features. We hypothesize that this observation may regard not only T cell responses to rh-Hsp60 but also to *M. bovis* Hsp65, rh-Hsp70, P180–188 *M. bovis* Hsp65 and P562–571 human Hsp60 derived peptides as we showed in several patients with established polyarticular JIA in our studied cohort.

## 3.2. Results to aim 2

### 3.2.1. Cell surface and relative gene expression of Hsp70 in human leukaemia cell lines

(published in *Hromadnikova I, Sedlackova L. Analysis of cell surface and relative gene expression of heat shock protein 70 in human leukemia cell lines. Leuk Lymphoma 2008;49(3): 570 – 576.*)

The goal of this part of study was to determine cell surface and mRNA expression of stress-inducible Hsp70 on leukaemia cell lines derived from patients with chronic myeloid leukaemia (Bcr-Abl positive K562 cells) and T-cell acute lymphoblastic leukaemia (Bcr-Abl negative Jurkat and CCRF-CEM cells). Hsp70 cell surface and mRNA expression was studied in K562, Jurkat and CCRF-CEM human leukaemia cell lines during several passages of in vitro culture by using flow cytometry and real-time quantitative RT-PCR.

Phenotypic analysis revealed that Hsp70 positivity on the plasma membrane of K562 cell line appeared approximately around the 61st day of the culture and reached the highest value on the day 72 (46.1%). Cell surface expression did not reach the cut-off from the beginning till the 42nd day of culture. A cut-off value for Hsp70 of 10% was chosen based on the results from previous screening of normal cells and tissues (peripheral blood lymphocytes and skin fibroblasts) by flow cytometry (Farkas et al., 2003; Nguyen et al., 2006). However, Jurkat and CCRF-CEM cell lines were Hsp70 membrane positive continuously from the beginning till the end of the culture and consequently monitored in the shorter cultivation period. The decrease of cell surface expression in these cell lines was observed with ongoing time of the culture. To compare cell surface with relative Hsp70 gene expression in leukaemia cell lines, RNA isolation was carried out at the same day of culture as flow cytometry acquisition. We found that mRNA expression in K562 cells was 9.88-fold higher in average when compared with healthy control. However, Hsp70 mRNA expression in Jurkat and CCRF-CEM cell lines was 0.47-fold and 0.62-fold lower in comparison with healthy control. Spearman's rank correlation was used to discover the strength of a link between two variables – Hsp70 cell surface and relative gene expression. Statistically significant strong negative correlation was found in case of Jurkat cell line. Weak positive correlation was observed in K562 and CCRF-CEM cell lines, however, the data did not reach the statistical significance.

The finding of positive cell surface Hsp70 expression in all of the leukaemia cell lines is in concordance with previous results describing frequent expression on the plasma membranes of tumour cells but not on normal cells (Multhoff et al., 1995; Shin et al., 2003; Gehrmann et al., 2002; Ferrarini et al., 1992; Hantschel et al., 2000; Gehrmann et al., 2003). The difference in cell surface Hsp70 expression among studied cell lines is probably due to the distinct types of leukaemia. Similar to other investigators we observed decreased membrane-bound Hsp70 expression in senescent and late-passage cells (Liu et al., 1989; Luce and Cristofalo, 1992; Effros et al., 1994).

We found increased mRNA expression only in K562 cells when compared with healthy control, though we observed positive cell surface Hsp70 expression in all cell lines. Hsp70 mRNA expression in Jurkat and CCRF-CEM cells was even lower in most analyses when compared with healthy control. We presume that highly increased cell surface expression together with non-elevated mRNA expression in Jurkat and CCRF-CEM cell lines could be clarified with the self-limiting control mechanism of overexpressed Hsp70 protein. It was showed that activated heat shock factor (HSF) associates with Hsp70 and suggested that Hsp70 may negatively regulate the activation of HSF (Abravaya et al., 1992). Ding et al.'s (Ding et al., 1998) data also suggested that overexpression of Hsp70 led to decreased Hsp70 gene transcription. The accumulated evidence suggested the presence of a negative feedback mechanism that prevented overproduction of Hsp70. Overexpressed Hsp70 was also found to inhibit the activation of HSF1, by activating protein phosphatase and inhibiting protein kinase C activity (Ding et al., 1998; Mosser et al., 1993; Kim et al., 1995). Other study previously showed that an increase in the degradation of Hsp70 mRNA was parallel to the accumulation of Hsp70. Later Hsp70 was found to bind to Hsp70 mRNA after stress and this interaction was suggested to be part of a self-limiting mechanism to reduce Hsp70 production (Balakrishnan and de Maio 2006). It seems that the cell surface expression in K562 cells changes according to the changes in mRNA expression during the cell culture. We suppose that the level of cell surface Hsp70 expression probably may not be sufficient to downregulate the mRNA expression.

### 3.2.2. Analysis of cell surface and relative gene expression of Hsp70 in RA patients

(published in *Sedlackova L, Nguyen TTH, Zlacka D, Sosna A, Hromadnikova I. Cell surface and relative mRNA expression of heat shock protein 70 in human synovial cells. Autoimmunity 2008 (in press)*).

Hsp70 cell surface and mRNA expression were studied in human fibroblast-like synovial cells, dermal fibroblasts and peripheral blood leukocytes derived from 24 RA patients, who underwent synovectomy by using flow cytometric analysis and real-time quantitative RT-PCR. For comparison, peripheral blood leukocytes of 17 healthy controls were tested. Since no control synovial tissues from non-inflammatory joint diseases like traumatic joint diseases or degenerative joint disease (osteoarthritis) were available to make comparison to chronic inflammatory arthritides (RA), the study was expanded for the data concerning autologous dermal fibroblasts derived from the operation wound of patients with RA.

Consistently with our previous studies (Nguyen et al., 2006; Hromadnikova et al., 2008) Hsp70 membrane positivity was detected on fibroblast-like synovial cells in 13 out of 18 RA patients (median 16.5%) in contrast to autologous or healthy control peripheral blood lymphocytes (RA patients: median 2.9%; healthy controls: median 4.5%) and/or autologous dermal fibroblasts (median 4.3%), which showed in most cases no Hsp70 membrane expression. Increased Hsp70 expression on fibroblast-like synovial cells when compared with autologous and/or healthy control peripheral blood lymphocytes ($P = 0.002$, $P = 0.002$) as well as autologous dermal fibroblasts ($P < 0.001$) was statistically significant. Healthy control peripheral blood lymphocytes were used as a negative control for Hsp70 cell surface expression studies. We observed strong Hsp70 cell surface expression on monocytes in all tested patients (median 58.1%) and healthy controls (median 47.5%) even if not statistically significant ($P = 0.52$). A significantly higher Hsp70 membrane expression was found on granulocytes of healthy controls (median 6.4%) when compared with granulocytes of RA patients ($P = 0.005$).

Significantly higher relative Hsp70 gene expression in RA-derived synovial cells (median 1.70) was observed when compared with autologous peripheral blood leukocytes (median 0.68, $P < 0.001$). However, we found significantly lower relative gene expression in peripheral blood leukocytes of RA patients in comparison with healthy controls (median 1.64, $P < 0.001$). On the other hand, Hsp70 gene expression compared between RA-derived

synovial cells and peripheral blood leukocytes of healthy controls did not reach any statistical significance (P = 0.83). Interestingly, we found that Hsp70 gene expression in RA non-affected skin dermis gained from the operation wound was 3.7-fold higher in average (median 8.30) when compared to autologous RA-affected synovial tissue (P < 0.001); 10.1-fold higher in average when compared to autologous peripheral blood leukocytes (P < 0.001) and 4.5-fold higher in average comparing to control peripheral blood leukocytes (P < 0.001).

Our finding concerning Hsp70 membrane positivity on fibroblast-like synovial cells in RA patients was consistent with the data of Schett et al. (Schett et al., 1998), who previously reported enhanced cytoplasmic expression of inducible Hsp70 in RA synovial tissue using Western blotting, immunohistochemistry and immunofluorescence. However, in another study the overexpression of constitutive heat shock cognate 70 (Hsc70) but not inducible Hsp70 was detected in RA synovial lining cells using immunochemistry and Western blotting (Schick et al., 2004).

Since many cell surface receptors like CD14, scavenger receptors (CD36, lectin-like oxidized low-density lipoprotein receptor (LOX-1), etc.), CD40, $\alpha$2-macroglobulin/- low-density lipoprotein receptor (LRP/CD91), Toll-like receptors (TLR-2 and TLR-4) and c-type lectins (CD94, etc.) that can bind Hsps (Calderwood et al., 2007) were described, we presume that we might detect extracellular Hsp70 bound to these receptors expressed on peripheral blood monocytes and granulocytes. To support this hypothesis, significantly higher levels of soluble inducible Hsp70 were detected in synovial fluids of RA patients. Further, soluble inducible Hsp70 was also detected in sera of RA patients as well as in healthy controls (Martin et al., 2003; Hromadnikova et al., 2008).

Hsp70 is the major stress-inducible Hsp, whose gene expression must be tightly regulated. However, the precise mechanism for control of Hsp70 expression has not been completely delineated.

Significantly higher Hsp70 gene expression observed in synovial cells of RA patients when compared with autologous peripheral blood leukocytes could support the observation of Hsp70 positive cell surface expression on synovial cells derived from RA-affected joints. However, the difference in Hsp70 gene expression between RA-derived synovial cells and healthy control peripheral blood leukocytes was not observed. We also found significantly lower relative gene expression in peripheral blood leukocytes of RA patients in comparison with healthy controls. We speculate that this observation might be explained by the administration of immunosuppressive therapy in a cohort of RA patients. However, Schett et al. (Schett et al., 1998) described that non-steroidal anti-inflammatory drugs triggered only an incomplete heat shock response, with HSF1 activation but not Hsp70 induction, whereas

steroids and immunosuppressive drugs did not affect the heat shock response at all. Interestingly, the highest Hsp70 gene expression was observed in RA non-affected skin dermis gained from the operation wound when compared to autologous RA-affected synovial tissue, autologous and/or control peripheral blood leukocytes. Skin as a barrier to the environment is much more exposed to various external factors, such as heat, UV light, microorganism, chemicals, etc., causing mainly apoptosis of epidermal keratinocytes. Some of them may affect even the deeper layers containing dermal fibroblasts. Hsp70 expression is important as a molecular repair factor that maintains the protective function of the normal skin. As a physiological response to potentially harmful environmental stress factors, these cells produce higher levels of Hsp70 comparing to the cells of internal organs and tissues (Trautinger et al., 1993). We speculate that the Hsp70 expression in skin dermis might be intensified also by the shock caused by the disinfection and sterile folio cover of the field of the operation, however, dermal fibroblasts were growing from the primary tissue very well in most patients.

We summarise that an increased Hsp70 gene expression in RA-affected synovial tissue is followed by Hsp70 cell surface expression on fibroblast-like synovial cells growing from RA synovial tissue. Hsp70 may be translocated to the cell surface from the cytosol and/or Hsp70 released from inflamed synovial tissue may be captured onto the membrane of synovial cells from the extracellular space via the CD91 receptor as we have reported recently (Hromadnikova et al., 2008). The significance of the Hsp70 presence on the cell surface of RA-affected synovial cells remains undefined but may take part in the development of a higher resistance to stress-induced apoptosis.

# 4 Conclusion

The importance of Hsps has been established by numerous studies concerning infection, inflammation, autoimmune disease and tumour immunity. Our research focused in particular on the supposed role of Hsps in pathogenesis of acute GvHD with simultaneous study of Hsps responses in actual and/or anamnestic infections. Similar Hsp study was also done in the cohort of JIA patients with the expansion for the responses against Hsp-derived synthetic peptides. Our another study targeted the cell surface and mRNA expression of Hsp70 in leukaemia cell lines and RA-affected synovial tissue. The summarized results are listed below.

❖ Significantly high proliferative response to rh-Hsp60 as well as *M. bovis* Hsp65 was demonstrated in a cohort of pretransplant patients with anamnestic and/or actual infection when compared with a cohort of patients without infection and healthy individuals. Strong PBMC responses to Hsps were found in patients who were at present colonized with *Escherichia coli* and *Klebsiella pneumoniae* or had previously *K. pneumoniae* infection with subsequent sepsis. Our findings support various studies dealing with immunodominant Hsps in connection with several pathogens and infectious diseases.

Although no statistical difference for proliferative response to PHA was observed, PBMC responses against all tested Hsps comparing a cohort of patients with acute GvHD and that with no sign of GvHD resulted in significantly lower SI for all tested Hsps in patients with acute GvHD. Lower stimulation with Hsps during acute GvHD might be explained by the stress-induced upregulation of self-Hsps synthesis that might lead to the inhibition of self-Hsps reactive T-cell response and that it might indicate the predominance of host-reactive donor $T_H1$ cell population and the lack and/or insufficient $T_H2$ and $T_{Reg}$ response resulting in local host tissue damage in the gut, liver and skin. We hypothesize that increased Hsp-specific stimulation may reflect the presence of protecting regulatory T cells preventing the development of $T_H1$-mediated diseases involving acute GvHD.

❖ Elevated proliferative response to rh-Hsp60, *M. bovis* Hsp65 and P562–571 human Hsp60 derived peptide was found in patients with JIA polyarthritis. Significantly elevated proliferation to P180–188 *M. bovis* Hsp65 was found in JIA lasting more than 2 years. None of the particular clinical characteristics (RF, ANA, HLA B27 and disease activity) seemed to

be associated with Hsp or Hsp-derived synthetic peptide proliferative response in the JIA cohort.

❖ Hsp70 was found to be expressed on the cell surface of K562, Jurkat and CCRF-CEM human leukaemia cell lines during several passages of in vitro culture. Even if all the cell lines were positive for cell surface Hsp70 expression, increased mRNA expression was observed only in K562 cells when compared with healthy controls. Our observation in this study raises the questions about the possible inhibition switch of the gene transcription in dependence on the elevation of the protein expression, which could explain the non-elevated mRNA expression in Jurkat and CCRF-CEM cell lines.

❖ Hsp70 gene expression in RA-affected synovial tissue is followed by Hsp70 cell surface expression on fibroblast-like synovial cells growing from RA synovial tissue. Significantly higher Hsp70 membrane positivity was found on fibroblast-like synovial cells in RA patients than on autologous and healthy control peripheral blood lymphocytes and/or autologous dermal fibroblasts. We also observed significantly higher Hsp70 gene expression in synovial cells of RA patients when compared with autologous peripheral blood leukocytes. Interestingly, we found that Hsp70 gene expression in RA non-affected skin dermis gained from the operation wound was higher when compared to autologous RA-affected synovial tissue. As a physiological response to potentially harmful environmental stress factors, skin dermis apparently produces higher levels of Hsp70 comparing to the cells of internal organs and tissues.

# 5 References


Abravaya K, Myers MP, Murphy SP, Morimoto RI. The human heat shock protein hsp70 interacts with HSF, the transcription factor that regulates heat shock gene expression. Genes Dev 1992;6:1153-1164.

Arnold D, Faath S, Rammensee H, Schild H. Cross-priming of minor histocompatibility antigen-specific cytotoxic T cells upon immunization with the heat shock protein gp96. J Exp Med 1995;182:885-9.

Balakrishnan K, de Maio A. Heat shock protein 70 binds its own messenger ribonucleic acid as part of a gene expression self-limiting mechanism. Cell Stress Chaperones 2006;11:44-50.

Calderwood SK, Mambula SS, Gray PJ Jr., Theriault JR. Extracellular heat shock proteins in cell signaling. FEBS Lett 2007;581:3689–94.

Cancino-Diaz ME, Perez-Salazar JE, Dominguez-Lopez L, et al. Antibody response to Klebsiella pneumoniae 60 kDa protein in familial and sporadic ankylosing spondylitis: role of HLA-B27 and characterization as a GroEL-like protein. J Rheumatol 1998;25:1756–1764.

D'Andrea AD, Bayever E, Haines KM, et al. Several evaluation of lymphocyte function in bone marrow-grafted patients. Diagn Immunol 1986;4:281–287.

Danieli MG, Markovits D, Gabrielli A, Corvetta A, Giorgi PL, van der Zee R et al. Juvenile rheumatoid arthritis patients manifest immune reactivity to the mycobacterial 65-kDa heat shock protein, to its 180–188 peptide, and to a partially homologous peptide of the proteoglycan link protein. Clin Immunol Immunopathol 1992;64:121–8.

De Graeff-Meeder ER, van der Zee R, Rijkers GT, Schuurman HJ, Kuis W, Bijlsma JWJ et al. Recognition of human 60 kDa heat shock protein bz mononuclear cells from patients with juvenile chronic arthritis. Lancet 1991;337:1368–72.

De Graeff-Meeder ER, van Eden W, Rijkers GT, et al. Juvenile chronic arthritis: T cell reactivity to human HSP60 in patients with a favorable course of arthritis. J Clin Invest 1995;95:934–40.

De Kleer IM, Kamphuis SM, Rijkers GT, Scholtens L, Gordon G, De Jager W et al. The spontaneous remission of juvenile idiopathic arthritis is characterized by CD30+ T cells directed to human heatshock protein 60 capable of producing the regulatory cytokine interleukin-10. Arthritis Rheum 2003;48:2001–10.

Ding XZ, Tsokos GC, Kiang JG. Overexpression of HSP-70 inhibits the phosphorylation of HSF1 by activating protein phosphatase and inhibiting protein kinase C activity. FASEB J 1998;12:451-459.

Dominguez-Lopez ML, Cancino-Diaz ME, Jimenez- Zamudio L, Granados-Arreola J, Burgos-Vargas R, Garcia-Latorre E. Cellular immune response to Klebsiella pneumoniae antigens in patients with HLA-B27+ ankylosing spondylitis. J Rheumatol 2000;27:1453–1460.

Effros RB, Zhu X, Walford RL. Stress response of senescent T lymphocytes: reduced hsp70 is independent of the proliferative block. J Gerontol 1994;49:B65-B70.

Farkas B, Hantschel M, Magyarlaki M, Becker B, Scherer K, Landthaler M, et al. Heat shock protein 70 membrane expression and melanoma-associated marker phenotype in primary and metastatic melanoma. Melanoma Res 2003;13:147-152.

Ferrarini M, Heltai S, Zocchi MR, Rugarli C. Unusual expression and localization of heat-shock proteins in human tumor cells. Int J Cancer 1992;51:613-619.

Gehrmann M, Pfister K, Hutzler P, Gastpar R, Margulis B, Multhoff G. Effects of antineoplastic agents on cytoplasmic and membrane-bound heat shock protein 70 (Hsp70) levels. Biol Chem 2002;383:1715-1725.

Gehrmann M, Schmetzer H, Eissner G, Haferlach T, Hiddenmann W, Multhoff G. Membrane-bound heat shock protein 70 (Hsp70) in acute myeloid leukemia: a tumor specific rocognition structure for the cytolytic activity of autologous NK cells. Haematologica 2003;88:474-476.

Hakim FT, Mackall CL. The immune system: effector and target of graft-versus-host disease. In: Ferrara JLM, Deeg HJ, Burakoff SJ, eds. Graft-vs.-Host Disease, 2nd edn. New York: Marcel Dekker, Inc., 1997: pp. 257–289.

Hantschel M, Pfister K, Jordan A, Scholz R, Andreesen R, Schmitz G, et al. Hsp70 plasma membrane expression on primary tumor biopsy material and bone marrow of leukemic patients. Cell Stress Chaperones 2000;5:438-442.

Hromadnikova I, Nguyen TTH, Zlacka D, Sedlackova L, Popelka S, Veigl D, Pech J, Vavrincova P, Sosna A. Expression of heat shock protein receptors on fibroblast-like synovial cells derived from rheumatoid arthritis-affected joints. Rheumatol Int 2008;28:837-44.

Jarvis M, Marzolini M, Wang XN, Jackson G, Sviland L, Dickinson AM. Heat shock protein 70: correlation of expression with degree of graft-versus-host response and clinical graft-versus-host disease. Transplantation 2003;76:849-853.

Jones DB, Coulson AF, Duff GW. Sequence homologies between hsp60 and autoantigens. Immunol Today 1993;14:115–8.

Kim D, Ouyang H, Li GC. Heat shock protein hsp70 accelerates the recovery of heat-shocked mammalian cells through its modulation of heat shock transcription factor HSF1. Proc Natl Acad Sci USA 1995;92:2126-2130.

Lamb JR, Bal V, Mendez-Samperio P, Mehlert A, So A, Rothbard J. Stress proteins may provide a link between the immune response to infection and autoimmunity. Int Immunol 1989;1:191-196.

Liu AY, Lin Z, Choi HS, Sorhage F, Li B. Attenuated induction of heat shock gene expression in aging diploid fibroblasts. J Biol Chem 1989;264:12037-12045.

Luce MC, Cristofalo VJ. Reduction in heat shock gene expression correlates with increased thermosensitivity in senescent human fibroblasts. Exp Cell Res 1992;202:9-16.

Martin CA, Carsons SE, Kowalewski R, Bernstein D, Valentino M, Santiago-Schwarz F. Aberrant extracellular and dendritic cell (DC) surface expression of heat shock protein (hsp) 70 in the rheumatoid joint: Possible mechanisms of hsp/DC-mediated cross-priming. J Immunol 2003;171:5736-5742.

Melnick J, Argon Y. Molecular chaperones and the biosynthesis of antigen receptors. Immunol Today 1995;16:243–250.

Mosser DD, Duchaine J, Massie B. The DNA-binding activity of the human heat shock transcription factor is regulated in vivo by hsp70. Mol Cell Biol 1993;13:5427-5438.

Multhoff G, Botzler C, Wiesnet M, Muller E, Meier T, Wilmanns W et al. A stress-inducible 72-kDa heat-shock protein (HSP72) is expressed on the surface of human tumor cells, but not on normal cells. Int J Cancer 1995;61:272-279.

Multhoff G, Pfister K, Gehrmann M, Hantschel M, Gross K, Hafner M et al. A 14-mer Hsp70 peptide stimulates natural killer (NK) cell activity. Cell Stress Chaperones 2001;6:337–44.

Nguyen TT, Gehrmann M, Zlacka D, Sosna A, Vavrincova P, Multhoff G, et al. Heat shock protein 70 membrane expression on fibroblast-like synovial cells derived from synovial tissue of patients with rheumatoid and juvenile idiopathic arthritis. Scand J Rheumatol 2006;35:447-453.

Prakken ABJ, van Eden W, Rijkers GT, et al. Autoreactivity to human heat-shock protein 60 predicts disease remission in oligoarticular juvenile rheumatoid arthritis. Arthritis Rheum 1996;39:1826–32.

Prakken B, Kuis W, van Eden W, Albani S. Heat shock proteins in juvenile idiopathic arthritis: keys for understanding remitting arthritis and candidate antigens for immune therapy. Curr Rheumatol Rep 2002;4:466–73.

Schett G, Redlich K, Xu Q, Bizan P, Groger M, Tohidast-Akrad M, et al. Enhanced expression of heat shock protein 70 (hsp70) and heat shock factor I (HSF1) activation in rheumatoid arthritis synovial tissue. Differential regulation of hsp70 expression and HSF1 activation in synovial fibroblasts by proinflammatory cytokines, shear stress, and antiinflammatory drugs. J Clin Invest 1998;102:302–311.

Schick C, Arbogast M, Lowka K, Rzepka R, Melchers I. Continuous enhanced expression of Hsc70 but not Hsp70 in rheumatoid arthritis synovial tissue. Arthritis Rheum 2004;50:88–93.

Shin BK, Wang H, Yim AM, Le Naour F, Brichory F, Jang JH et al. Global profiling of the cell surface proteome of cancer cells uncovers an abundance of proteins with chaperone function. J Biol Chem 2003;278:7607-7616.

Shinnick TM, Vodkin MH, Williams JC. The Mycobacterium tuberculosis 65-kilodalton antigen is a heat shock protein which corresponds to common antigen and to the Escherichia coli GroEL protein. Infect Immun 1988;56:446-451.

Shinnick TM. Heat shock proteins as antigens of bacterial and parasitic pathogens. Curr Top Microbiol Immunol 1991;167:145-160.

Suto R, Srivastava PK. A mechanism for the specific immunogenicity of heat shock protein-chaperoned peptides. Science 1995;269:1585–1588.

Trautinger F, Trautinger I, Kindas-Mu¨gge I, Metze D, Luger TA. Human keratinocytes in vivo and in vitro constitutively express the 72-kD heat shock protein. J Invest Dermatol 1993;101:334–338.

Van Eden W, van der Zee R, Paul AG, Prakken BJ, Wendling U, Anderton SM, Wauben MH. Do heat shock proteins control the balance of T-cell regulation in inflammatory diseases? Immunol Today 1998;19:303-7.

Young D, Lathigra R, Hendrix R, Sweetser D, Young RA. Stress proteins are immune targets in leprosy and tuberculosis. Proc Natl Acad Sci USA 1988;85:4267–4270.

Zügel U, Kaufmann SH. Role of heat shock proteins in protection from and pathogenesis of infectious diseases. Clin Microbiol Rev 1999;12:19-39.

# 6 List of author's publications

**Sedlackova L**, Sedlacek P, Hromadnikova I. Peripheral blood mononuclear cell responses to heat shock proteins in patients undergoing stem cell transplantation. Pediatr Transplantation 2006:10:178-186. **IF = 1.505**

**Sedlackova L**, Velek J, Vavrincova P, Hromadnikova I. Peripheral blood mononuclear cell responses to heat shock proteins and its derived synthetic peptides in juvenile idiopathic arthritis patients. Inflamm Res 2006: 55:153-9. **IF = 1.504**

Hromadnikova I, **Sedlackova L**. Analysis of cell surface and relative gene expression of heat shock protein 70 in human leukemia cell lines. Leuk Lymphoma 2008; 49(3): 570 – 576. **IF = 1.512**

**Sedlackova L**, Nguyen TTH, Zlacka D, Sosna A, Hromadnikova I. Cell surface and relative mRNA expression of heat shock protein 70 in human synovial cells. Autoimmunity 2008 (in press). **IF = 2.887**

Hromadnikova I, Nguyen TT, Zlacka D, **Sedlackova L**, Popelka S, Veigl D, Pech J, Vavrincova P, Sosna A. Expression of heat shock protein receptors on fibroblast-like synovial cells derived from rheumatoid arthritis-affected joints. Rheumatol Int 2008;28:837-44. **IF = 1.27**

Hromadnikova I, Zlacka D, Nguyen TT, **Sedlackova L**, Zejskova L, Sosna A. Fetal cells of mesenchymal origin in cultures derived from synovial tissue and skin of patients with rheumatoid arthritis. Joint Bone Spine 2008 (in press). **IF = 1.659**

Hromadnikova I, Zlacka D, Nguyen TT, **Sedlackova L**, Zejskova L, Sosna A. Fetal cells of mesenchymal origin in cultures derived from synovial tissue and skin of patients with rheumatoid arthritis. Rev Rhum 2008 (in press). **IF = 0.624**