Chapter 3

HSP70-specific immune responses are anti-inflammatory and inhibit proteoglycan-induced arthritis

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Abstract

Objective. Immune responses to heat shock proteins (HSPs) can be involved in the suppressive regulation of various inflammatory autoimmune diseases. The aim of this study was to explore the capacity of immune responses to HSP70 to prevent or arrest inflammatory damage in an antigen-induced arthritis model and to search for the mechanisms involved.

Methods. The anti-inflammatory effect of a single dose of microbial (mycobacterial) HSP70 in adjuvant (DDA) was investigated in proteoglycan (PG)-induced arthritis (PGIA), a progressive autoimmune murine model of rheumatoid arthritis (RA).

Results. HSP70 pretreatment significantly delayed arthritis onset and dramatically reduced severity. Joint sections of HSP70-pretreated arthritic mice showed very mild leukocyte infiltration, less reactive synovial cell proliferation, and consequently almost no cartilage damage compared to the joints of control animals. The protective effect of HSP70 was accompanied with increased HSP70-specific and PG-specific T cell proliferation, IFNγ and IL-10 production. Interestingly, not only after restimulation in vitro with HSP70, but also with PG enhanced IL-10 production was observed in animals that were protected by the administration of HSP70.

Conclusion. HSP70 immunization can suppress the development of inflammation and subsequent tissue damage in PGIA. Moreover, HSP70 treatment results in an altered immune response as shown by proliferation and a regulatory cytokine profile to both HSP70 and PG. Together these data demonstrate the therapeutic potential of HSP70 in arthritis via the induction of IL-10.
**Introduction**

Heat shock proteins (HSPs), present in all cellular organisms, both prokaryotic and eukaryotic, function as chaperones in intracellular protein folding, assembly and transport, and are classified into families on the basis of their molecular weight. HSP families are highly conserved throughout evolution (1-3), and some mammalian family members have extensive homologies with bacterial HSPs (4,5). HSPs, also called “stress-proteins”, become markedly over-expressed by cells in the synovium during inflammation (4,6-8). A variety of different stressors (e.g. proinflammatory cytokines) is present in the inflamed synovial tissue of rheumatoid arthritis (RA) patients, each of which has the potential to induce HSP upregulation and unleash an HSP-specific immune response (7,9,10). HSPs are highly immunogenic and have the potential to trigger immunoregulatory pathways (11). Increasing evidence suggests that T cells which react against HSPs inhibit immune responses, serving as a feedback loop to suppress an appropriate immune response. More specifically, immunity to HSPs can suppress immune responses that occur in various inflammatory conditions, such as in RA, juvenile idiopathic arthritis (JIA) and related disorders (11-14).

Although HSPs have well described roles as chaperones for intracellular proteins, the significance of the immunomodulatory capacity of HSPs in human arthritis is only now becoming clear (15-17). Much of the evidence for the immunomodulatory capacity of HSPs in inflammatory diseases comes from analysis of experimental models (5,11,18), in vitro study’s on HSP60 in patients with autoimmune diseases (14,17) and a few clinical trials in patients with HSP peptides (19,20). Less is known about HSP70, one of the most conserved HSPs (1).

Earlier, we have shown that microbial HSP70 can trigger T cells cross-reactive with self-HSP which then down-regulate adjuvant arthritis via interleukin (IL)-10 release (21,22). More recently, Corrigall et al. (16) demonstrated that, the stress-inducible immunoglobulin binding protein, BiP (member of the HSP70 family) could trigger the production of anti-inflammatory cytokines in RA peripheral blood mononuclear cells (PBMCs). An increased response to BiP was observed in synovial joints of RA patients, indicating attempts of the immune system to regulate the ongoing inflammation (23). Overall, proinflammatory cytokines do promote HSP upregulation (7,9), and such upregulated HSP may promote a counterregulatory, suppressive immune response in PBMCs or synovial fluid derived mononuclear cells. As non-steroid anti-inflammatory drugs (NSAIDs) (24) and gold (25) can also induce the expression of HSP70, these compounds might have added value in promoting HSP-directed immunoregulation.

Thus, HSP70 may be a promising target for immunoregulation and may offer opportunities for novel therapies for chronic inflammatory arthritis.

The aim of this study was to further analyze the anti-inflammatory mechanism of HSP70-induced immune regulation in arthritis. We investigated the protective effects of HSP70 in proteoglycan-induced arthritis (PGIA), a progressive T cell-dependent, antibody-mediated autoimmune murine model of RA (26). The progressive character and high incidence of PGIA make this model optimal for testing immunomodulatory agents (26,27). Moreover, in this model, we can induce autoimmune inflammatory arthritis using human cartilage PG (hPG) mixed with synthetic adjuvant dimethyldioctadecylammonium bromide (DDA) instead of complete Freund’s adjuvant (CFA) (27). Therefore, we can exclude the role of interfering immune responses induced by microbial HSPs present in CFA.
In the present study we show that HSP70 pretreatment dramatically suppresses the development of inflammation and subsequent tissue damage in PGIA. Moreover, we demonstrate that HSP70 pretreatment results in a regulatory immune response not only to HSP70 but also to hPG.
Materials and Methods

Antigens, animals and immunization
The use of human cartilage from joint replacement surgeries for antigen isolation was approved by the Institutional Review Board of the Rush University Medical Center (Chicago, USA) and the medical ethical regulations of the University Medical Center Utrecht (Utrecht, the Netherlands). All animal experiments were approved by the Institutional Animal Care and Use Committee of the Rush University Medical Center (Chicago, USA) and by the Animal Experiment Committee of Utrecht University (Utrecht, the Netherlands).

Female BALB/c mice at the age of 16-26 weeks (Charles River, Sulzfeld, Germany) were injected intraperitoneally (IP) with recombinant HSP70 of Mycobacterium tuberculosis (Mt) (100 µl of 1 mg/ml; obtained from LIONEX Diagnostics & Therapeutics GmbH, Braunschweig, Germany), or with recombinant enhanced green fluorescent protein (EGFP) (100 µl of 1 mg/ml; control protein produced in E.coli in a similar way as HSP70) both mixed 1:1 v/v with the synthetic adjuvant DDA (Sigma, Zwijndrecht, the Netherlands; 20 mg/ml emulsified in phosphate buffered saline (PBS)) or with PBS. HSP70, EGFP or PBS were injected IP 10 days prior to the first immunization for arthritis (day -10). Arthritis was induced with human cartilage proteoglycan (hPG) using a standard immunization protocol as described (27,28). Briefly, the antigen injection (100 µg hPG protein in 100 µl PBS) was given IP with 2 mg of DDA (in 100 µl PBS) on days 0 and 21. Mice were sacrificed on day 35 or in separate experiments on day 63.

Assessment of arthritis
Paws of mice were examined 3 times a week to record arthritic changes of the joints. The onset and severity of arthritis were determined using a visual scoring system based on swelling and redness of paws as described (27,28). In brief, the degree of joint swelling for each paw (scored from 0 to 4) was used to express a total arthritis score, with a possible maximum severity index of 16 per animal. The first clinical appearance of swelling was recorded as the onset of arthritis (26,28). As above, mice were sacrificed on day 35 following the first hPG/DDA immunization, joints fixed in 10% buffered formalin, decalcified in 0.33 M neutralized EDTA, embedded in paraffin and 5 µm sagittal sections were stained with hematoxylin and eosin.

Measurement of antigen-specific T cell responses
Single-cell suspensions of spleens were cultured in triplicates in 96-well flat bottom plates (Corning B.V. Life Sciences, Schiphol-Rijk, the Netherlands) at 2 x 10^6 cells per well, in the presence or absence of HSP70 (10 µg/ml), hPG (10 µg/ml) or ovalbumin (OVA; 10 µg/ml). Concanavalin A (ConA; 2 µg/ml) was used as a positive control for T cell proliferation. Iscove’s Modified Dulbecco’s Medium (IMDM) supplemented with 10% FBS (Bodinco B.V., Alkmaar, the Netherlands), 2mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 5 x 10^{-5} M 2-mercaptoethanol was used as culture medium. After 96 h, the cells were pulsed overnight with [H]-thymidine (0.4 µCi per well; Amersham Biosciences Europe GmbH, Roosendaal, the Netherlands), harvested and uptake was measured by liquid scintillation counting (Microbeta, Perkin-Elmer Inc., Boston, MA). The magnitude of the
proliferative response was expressed as delta counts per minute (Δ cpm) calculated by subtracting the cpm of non-stimulated from cpm of stimulated cultures.

In separate experiments for in vitro epitope mapping, the responses to the 123 overlapping 15-mer peptides (20 µg/ml), covering HSP70 of Mycobacterium tuberculosis (21) were measured in triplicates using the same culturing condition, but using pooled lymph node cells of major draining lymph nodes obtained 12 days after IP immunization with HSP70 in DDA only. The peptides were synthesized via automated simultaneous multiple peptide synthesis developed with a standard autosampler (Gilson 221, Gilson Medical Electronics SA, Villiers le Bel, France) as described previously (29). Briefly, standard Fmoc chemistry with in situ PyBop/NMM activation of the amino acids in a 5-fold molar excess with respect to 2 µmol reactive equivalents per peptide on the PAL-PEG-PS resin (Perseptive Biosystems, Foster City, CA, USA) was employed. Peptides were obtained as C-terminal amides after cleavage with 90-95% trifluoroacetic acid/scavenger cocktails. Peptides were analysed by reversed-phase high-performance liquid chromatography and checked via electrospray ionisation mass spectrometry (LCQ; Thermoquest, Breda, The Netherlands). Purity of the peptides ranged between 50 to 90 %. Sequences of predominantly recognized peptides are shown in Table 1.

Cytokine measurement by multiplex analysis

Supernatants of antigen stimulated spleen cell cultures were collected for cytokine assays after 72 h and analyzed for IL-10 and interferon-gamma (IFNγ) simultaneously using the Luminex 100 system (Becton Dickinson, Mountain View, CA). The LINCOplex assay was performed according to the manufacturer’s instructions (Linco Research, Inc., St. Charles, Missouri). In brief, the antibody coated microspheres were incubated with standards, controls, and samples (25 µl) in a 96-well microtiter filter plate overnight at 4ºC. Plates were washed and the mixture of biotinylated IL-10 and IFNγ detection antibodies (Lincoplex) was added for 30 min at room temperature. After repeated washing, streptavidin-phycoerythrin (PE) (Lincoplex) was added for an additional 30 min. Beads were exhaustively washed, resuspended and fluorescence intensity was read on the Luminex model 100 instrument. The concentrations of IL-10 and IFNγ in supernatants were calculated using LMAT software (Luminex Corporation, Austin, TX).
Table 1. Alignment of Mt HSP70 epitope sequences and homologous mouse HSP70 sequences.

<table>
<thead>
<tr>
<th>HSP70 epitope</th>
<th>Amino Acid Sequence</th>
<th>Mean Δ cpm ± SEM</th>
</tr>
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<tbody>
<tr>
<td>P5-6 (22-40)†</td>
<td>GDPVVANSEGRTTPSIV</td>
<td>4312 ± 2248 / 3727 ± 2475</td>
</tr>
<tr>
<td>Mouse GRP75</td>
<td>KQAkJkekaNSGVTSV</td>
<td></td>
</tr>
<tr>
<td>P10-11 (46-65)</td>
<td>GEVLVGGPAAPNQVAVNDVT</td>
<td>3903 ± 1620 / 4831 ± 2142</td>
</tr>
<tr>
<td>Mouse GRP75</td>
<td>GERLVGMPAQRQAVTNPNT</td>
<td></td>
</tr>
<tr>
<td>P25-26 (121-140)</td>
<td>YFNDAGQATKDAQQIAGLN</td>
<td>5518 ± 983 / 4832 ± 4159</td>
</tr>
<tr>
<td>Mouse BiP (GRP78)</td>
<td>YFNDAGQATKDAQQIAGLN</td>
<td></td>
</tr>
<tr>
<td>P90 (445-459)</td>
<td>QIEVTFIDANGIVH</td>
<td>4167 ± 1798</td>
</tr>
<tr>
<td>Mouse GRP75</td>
<td>QIEVTFIDANGIVH</td>
<td></td>
</tr>
<tr>
<td>P94-95 (464-484)</td>
<td>DKGKGRIIRIQEGSSKE</td>
<td>4539 ± 3666 / 4553 ± 2675</td>
</tr>
<tr>
<td>Mouse GRP75</td>
<td>DKGKGGRQIVIQGSGKL</td>
<td></td>
</tr>
<tr>
<td>P107-108 (530-549)‡</td>
<td>AEGGSKVPEDTLNKVDAAVA</td>
<td>4409 ± 3449 / 5197 ± 3839</td>
</tr>
<tr>
<td>P118 (585-599)‡</td>
<td>AQAASQATGAAPGG</td>
<td>4242 ± 2367</td>
</tr>
<tr>
<td>P120 (596-610)‡</td>
<td>HP GGEPGGAHF GSAD</td>
<td>5250 ± 2048</td>
</tr>
</tbody>
</table>

Alignment of Mt HSP70 (Swissprot P0A5B9) sequences covering the predominately recognized peptides with the homologous sequences of the mouse HSP70 family members having the highest number of identical residues. Identical amino acids in both sequences are interconnected by lines, and conserved substitutions are interconnected by dots. HSP70 family members GRP75 (Swissprot P38647) and BiP/GRP78 (P20029) showed the highest degree of homology with the peptides recognized by Mt HSP70 immunized mice. Major epitopes and flanking peptides which also induced responses are shown. All other peptides were negative, e.g. had a mean Δ cpm of less than 3600, and data are not shown. The mean background values (responses without antigen) were 7260 ± 2242. † Within brackets residue numbers of Mt HSP70. ‡ No significant homologies were found.

Cytokine measurement by flow cytometry

Spleen cell suspensions of HSP70-pretreated and PBS control mice were cultured in the presence of HSP70 or hPG for 72 h to which for the last 4 h Brefeldin A (Sigma) was added. Cells were washed in PBS with 0.5% BSA and 0.01% sodium azide (FACS buffer) and stained with allophycocyanin (APC)-conjugated anti-CD4 or control mAbs (BD Biosciences Pharmingen, San Diego, CA) for 30 min on ice. Subsequently, cells were fixed (Cytofix/Cytoperm; BD Biosciences Pharmingen, San Diego, CA), permeabilized (Perm/Wash; BD Biosciences Pharmingen, San Diego, CA) and stained with PE-labeled anti-IL-10 and FITC-labeled anti-IFNγ for 30 min on ice. Cells were analyzed using a FACSCalibur flow cytometer and Cell Quest software (BD Biosciences Immunocytometry, San Jose, CA).

Statistical analysis

Unless stated otherwise, data are expressed as mean ± standard error of the mean (SEM). Statistical analysis of the arthritis score and disease incidence were carried out using the Mann-Whitney U test (two-tailed) using Prism software (version 3.00, Graphpad Software Inc., San Diego). The Student’s t-test was used for the analysis of antigen-specific proliferation. Significance level was set at (p < 0.05).
Results

Disease inhibitory effect of HSP70 on PGIA

The potential for HSP70 to ameliorate PGIA was examined by pretreatment of mice with microbial HSP70 in synthetic adjuvant DDA. Mice were injected with 100 µg HSP70 (group C) 10 days prior to the first hPG immunization (day -10), whereas control groups received EGFP (group B) or PBS (group A). On day 0 and 21, all mice were immunized with hPG in DDA to induce arthritis (Fig. 1A). HSP70 injected mice showed a 2-3-week delayed onset of arthritis and lower incidence compared with EGFP- or PBS-pretreated mice (Fig. 1B). Mean time of onset in HSP70-pretreated animals was at day 52 ± 3.2 compared with day 31 ± 2.0 in EGFP and 30 ± 1.0 in PBS pretreated mice (p<0.01). Furthermore, HSP70 significantly reduced severity of PGIA with a maximum arthritis score of 1.1 ± 0.3 compared with a score 4.6 ± 1.6 in the EGFP and 4.4 ± 1.2 in the PBS control mice; p<0.05 (Fig. 1C).

Figure 1. Schematics of the experimental groups and immunization (A), incidence (B) and severity (C-D) of hPG immunized BALB/c mice. Mice were injected intraperitoneally with either 100 µg of recombinant microbial HSP70 (group C) or 100 µg EGFP (group B) both emulsified in synthetic adjuvant DDA or PBS (group A) 10 days prior to the induction of arthritis. Arthritis was induced by two immunizations of hPG in DDA on days 0 and 21 and animals as described in methods. A significantly lower incidence (B) and less severe PGIA (C-D) were found in mice pretreated with HSP70. Incidence of arthritis was expressed as the cumulative percentage of arthritic animals (n = 10 in each group). The severity of the disease is shown as the maximum (highest) score per mouse at any time during disease (C; horizontal lines indicate the mean, significant differences, p<0.05 Student’s t-test, are indicated by asterisks) and as the mean arthritis score of arthritic animals only (D). Results are presented as the mean ± SEM, and are representative for 3 independent experiments.
The suppression of arthritis was complete (Fig. 1B and D) until day 42; thereafter some mild clinical symptoms occurred in a few HSP70-pretreated animals (Fig. 1D). In association with low severity of arthritis, joint sections of HSP70-pretreated arthritic mice showed very mild leukocyte infiltration, less reactive synovial cell proliferation, and consequently almost no cartilage damage compared to the joints of control animals (Fig. 2).

**Figure 2.** Histology of tarso-metatarsal joints of BALB/c mice pretreated with PBS (A) or with recombinant HSP70 (B). Mice received PBS or HSP70/DDA treatment 10 days prior to the immunization with hPG/DDA to induce arthritis. These animals were sacrificed on day 35 after the first hPG/DDA injection for histology. Massive synovial hyperplasia with infiltrating leukocytes, and cartilage (arrow heads) and bone erosions (arrows) can be seen in mice that received only PBS. Synovium of joint sections of HSP70-pretreated mice showed only a very few leukocytes, and no synovial cell proliferation or cartilage destruction can be seen. Sections were stained with hematoxylin and eosin.

**Increased proliferative responses after immunization with HSP70**

To analyze the T cell response induced by administration of HSP70 we measured antigen-specific T cell proliferation in the HSP70-pretreated mice (Fig. 1, group C) and the PBS pretreated controls (disease control; Fig. 1, group A). For this purpose, splenocytes were harvested on day 35 and stimulated with ConA, HSP70, hPG or OVA. HSP70 treated animals showed a significantly enhanced response to HSP70, compared to the control group (Fig. 3). Remarkably, in the HSP70 group also a significant increased proliferative response was seen against the arthritis inducing hPG. This increased response was not seen for control antigen OVA or ConA.
Antigen-induced cytokine production after immunization with HSP70

Next, we analyzed the antigen-induced production of IL-10 and IFNγ of spleen cells (day 35) from HSP70-pretreated mice and PBS pretreated controls after in vitro stimulation with HSP70 or hPG. After 72h of culture, supernatants were collected and the IL-10 and IFNγ secretion was determined using multiplex analysis. Interestingly, not only HSP70-stimulated spleen cells but also cells stimulated with the arthritis inducing hPG were found to produce IL-10 in HSP70-pretreated animals, whereas the IL-10 level in PBS mice was significantly lower (Fig. 4A). Also the amount of INFγ produced by HSP70- and hPG-specific cells was significantly increased in the HSP70-pretreated mice (Fig. 4B). Consistent with the supernatant analysis, intra-cellular staining after 72 h in vitro stimulation with HSP70 or hPG, showed that slightly more IL-10 producing cells were present in HSP70-pretreated mice than in PBS-pretreated mice, not only after stimulation with HSP70 but also after hPG stimulation (Fig. 4C). This increased IL-10 expression was especially seen in the CD4+ cell population (1.4%). Also the number of IFNγ producing cells was increased in HSP70-pretreated mice compared to the PBS mice, but this was mainly in the CD4+ cell population (Fig. 4D).

Mapping of the T cell epitopes within HSP70

For the identification of T cell epitopes in Mycobacterium tuberculosis HSP70, a panel of 123 overlapping peptides (15 amino acids long) covering the complete sequence of HSP70 was tested for the ability to stimulate splenic (data not shown) and lymph node T cells from HSP70/DDA immunized mice harvested 12 days after immunization. T cells of HSP70 immunized mice predominantly recognized the peptides p5-6, p10-11, p25-26, p90, p94-95, p107-108, p118 and p120 of HSP70 (Table 1). Besides responses to the Mt HSP70-specific peptides (p107-108, p118 and p120) for which no homology with a mouse HSP70 was found, most of the predominantly recognized Mt HSP70 peptides were located especially in regions with a high degree of identity between Mt HSP70 and mouse HSP70 family members (Table 1). Interestingly, in the case of peptide p25 or p26 and peptide p90, 14 and 15 amino acids respectively, in the 15-mer peptide were identical with the mouse HSP70 homologue.
Role of HSP70-specific immune responses in PG-induced arthritis

Figure 4. In vitro cytokine secretion in antigen (HSP70 and hPG)-stimulated spleen cell cultures (A-B) and corresponding results of intracellular cytokine expression (IL-10 and IFNγ). Mice received PBS or recombinant HSP70 pretreatment 10 days prior to hPG/DDA immunizations and were sacrificed on experimental day 35 (as shown in Fig. 1A). Spleen cells were cultured in the presence of HSP70 or hPG and 72-h supernatants were harvested and assayed by multiplex analysis for IL-10 and IFNγ. Values represent the mean ± SEM of cytokine levels (n=8 mice in each group). Significant difference (p<0.05, Mann-Whitney U Test) is indicated by the asterisk. For flow cytometry (C-D) viable lymphocytes were gated, and IL-10 and IFNγ expressions of CD4+ cells are shown. The percentage of cells in each quadrant is indicated, and the numbers within brackets the percentage of CD4+ and CD4- cells. Results of a representative experiment are shown.
Discussion

Microbial HSPs are immunologically dominant antigens, despite the high degrees of sequence identity with mammalian (self)-HSP homologues (30). Recent studies showed that HSPs are critical antigens in the immune regulation of certain chronic inflammatory diseases, and are important in protection from disease (11,31). Initial clinical trials with a peptide of HSP60 in patients with type 1 diabetes (20) and with peptide DnaJp1 (E. coli HSP40) in RA (19,32) have indicated that HSPs contain important immunomodulatory epitopes which may be used for immunotherapy.

For further development of HSPs for therapy, it is essential to understand how and which immunomodulatory epitopes may affect chronic inflammatory arthritis. HSP70 might be a candidate antigen for immunoregulation for several reasons. It is a conserved immunogenic protein with the capacity to induce a self-HSP cross-reactive immune response, it is highly induced by stress; it becomes upregulated in the synovium of arthritis patients and it is an immune target in RA (4,7,16,23). While the mechanism of immunoregulation is not exactly known, we propose that increased exposure to microbial HSP70 (in this case by immunization) leads to the activation of self-HSP70-reactive T cells that exert immunoregulatory activities towards self-HSP70-expressing inflamed synovial tissues (11,33).

RA is the result of a complex immunological process that involves both adaptive and innate immunity (34,35). PGIA is a T cell-dependent, antibody-supported chronic autoimmune murine model of RA (26), and can be induced without the use of CFA (27). In this study we demonstrated that HSP70 pretreatment resulted in a significant delay of the arthritis onset and dramatically reduced disease severity both clinically and histologically. In vitro tests showed increased proliferation to HSP70 in the HSP70-pretreated animals compared to the control group, indicating that the immunization procedure had been successful. Unexpectedly, in the HSP70-pretreated animals also an increased proliferative response was seen against the arthritis inducing hPG. HSP70 might have interacted with antigen presenting cells resulting in enhanced antigen priming to the subsequent hPG immunization. Srivastava and colleagues discovered that HSP70 potently stimulated Ag-specific T cell responses (36). Microbial HSP70 has been shown to exert a potent adjuvant effect in models of infection and solid tumors (37). In contrast to other studies in which arthritis is induced with avridine or CFA (22,38,39), this is the first time that in a (self)-antigen-induced arthritis model the anti-inflammatory capacity of HSP70 was studied. For this reason the increased proliferative response to the disease inciting antigen has not been noted so far and needs to be analyzed further. Especially that such increased proliferation can coincide with suppression of disease and production of IL-10 (see hereunder) needs further attention.

Pretreatment with HSP70 was seen to lead to production of IL-10 and IFNγ in spleen cells upon in vitro restimulation with HSP70. Interestingly, IL-10 and IFNγ were also found after restimulation with hPG in animals that were protected by the administration of HSP70. Similar observations have been made for HSP60 in JIA patients with a remitting form of disease. In these patients responses to HSP60 are consistent with a benign clinical course of this subgroup (14). These HSP60-specific T cells in oligoarticular JIA patients have the phenotype of human Tr1 cells (simultaneously producing IFNγ and IL-10) (40). It was shown that Tr1 clones can suppress the immune responses of other T cells in vitro and in vivo, including inhibiting the development of chronic inflammation and Th1-mediated
autoimmune diseases (41,42). Interestingly, also in human clinical infections, there are first reports of the presence of Tr1-like IL-10 and IFNγ double-producing T cells functioning as regulators of the antiparasite response while preventing hyperinflammation (43). Alternatively, IFNγ is a prototype Th1 proinflammatory cytokine (44). Therefore, the ratio of IL-10 to IFNγ can be seen as a measure for the balance of T-regulatory and Th1 cells. We recorded a raised IL-10 to IFNγ ratio against hPG in HSP70-pretreated mice (ratio in control mice 0.4; ratio in HSP70-pretreated mice 1.3). This suggests that the anti-inflammatory response induced by HSP70 in arthritis was IL-10 mediated. It is possible that HSP70-specific cells as well as hPG reactive cells, displaying a regulatory phenotype, influenced neighboring harmful autoreactive T cells, either directly or indirectly through cytokines; the locally produced anti-inflammatory cytokines may have modified effector T cell cytokine secretion, and may have switched the cytokine profile of hPG-specific cells into a regulatory IL-10 producing phenotype. The possible role of IL-10 in PGIA was indicated by the findings that in IL-10−/− mice the development of PGIA was dramatically more severe as compared to wild-type BALB/c mice (26). Moreover, Guichelaar et al. showed suppression of PGIA, upon in vivo transfer, of IL-10 producing hPG-specific T cells (in preparation). Thus, in PGIA IL-10 seems to be a prominent factor in the regulation of the disease and the protective HSP70 pretreatment is also likely to depend on IL-10 production.

In patients with RA, IL-10 is produced by synovial lining cells and high levels of IL-10 can be found in the synovial fluid (45,46). It has been shown that blocking of IL-10 in synovial membrane cultures from RA patients markedly increased the levels of IL-1 and TNFα, suggesting an inhibitory effect of IL-10 on pro-inflammatory cytokines in RA joints (45). It was observed that IL-10 stimulated PG synthesis and reversed cartilage degradation induced by activated mononuclear cells (47). IL-10 also correlated with diminished progression of joint destruction in RA (48). Moreover, IL-10 production has been described as being the most prominent characteristic of a subset of regulatory T cells (Tr1 cells) generated in the presence of IL-10 (41). Also in humans, the importance of IL-10 for the in vivo function of regulatory T cells has now positioned IL-10 as a crucial cytokine in the control of immune responses (42). As self-HSP70 is upregulated in joints of RA patients (7,23), the microbial HSP70 activated cells are possibly targeted to the joints on the basis of cross-reactivity with self-HSP70 (11). Increased and targeted HSP-specific immune responses might contribute to disease remission and restore a local balance between protective regulator and disease producing effector cells. This would be in line with what was noted earlier by Kimura et al. (49) that T cells that recognize a microbial HSP70 epitope play a regulatory role in inflammation during Listeria infection via the production of suppressive cytokines including IL-10. Furthermore, Detanico et al. (50) have recently observed that synovia-derived monocytes produce IL-10 in the presence of mycobacterial HSP70. Our present findings confirm previous reports on a protective effect of microbial HSP70 and BiP in rat adjuvant arthritis (21-23,39), and are well in line with the known protective effects of microbial HSP60 and microbial HSP10 (11).

As argued above the immunoregulatory qualities of HSPs may be due to their cross-reactivity between microbial HSPs and self-HSPs (21). Given the presence of T cell responses towards highly conserved or even identical sequences of HSP70 (see results) one may assume that following HSP70 immunization cross-reactive responses to self-HSP70 were induced. Thus, immunization with microbial HSP70 may have activated and expanded self-HSP70-reactive T cells.
Taken together we showed that HSP70 can modulate the development of inflammation in the PGIA model likely through IL-10 dependent mechanisms. The fact that HSP70-mediated preventive and therapeutic immune interventions are effective in animal models of chronic inflammatory diseases suggests the immunotherapeutic potential of HSP70 in patients with inflammatory autoimmune arthritis.

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References


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