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Strain Differences and the Role for HSP47 and HSP70 in Adjuvant Arthritis in Rats

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Abstract

Because of high sequence homology between microbial and endogenous heat shock proteins (HSP), immunological cross-reactivity to microbial HSP has been suggested as a possible cause of the development of autoimmune diseases, such as rheumatoid arthritis. The present study aimed to determine a potential role of HSP47, a molecular chaperone involved in the synthesis and assembly of collagen molecules, and microbial HSP71 (mHSP71) in adjuvant arthritis (AA) in two rat strains: Dark Agouti (DA), susceptible to AA induction and Albino Oxford (AO), which is resistant to AA induction. Immunization with complete Freund's adjuvant (CFA) induced an increased expression of HSP47 in joints of DA rats, which exhibited severe clinical signs of AA at the time of disease peak, while this protein was not detectable in joints of AO rats. In contrast, no strain differences in HSP72 (rat analogue of mHSP71) expressions in joints were observed. The increased levels of anti-HSP47 antibodies were detected in sera of DA rats during the AA peak, while the immunization with CFA increased levels of anti-mHSP71 antibodies in sera of AO rats. HSP47 and mHSP71 reduced proliferation of draining inguinal lymph node cells (LNC) in resistant AO rat strain, leading to a hypothesis that both HSP participated in AA control. Finally, mHSP71 potentiated the apoptotic response of LNC in susceptible DA rat strain. In conclusion, our findings indicate involvement of HSP47 in the development of AA in the rat, and point out to the regulatory role for both HSP47 and mHSP71.

Introduction

Heat shock proteins (HSP) are among the most abundant and evolutionary highly conserved molecules expressed in both prokaryotic and eukaryotic cells that perform numerous and specific functions in the organism. Their molecular chaperone function involves assisting in correct folding of newly synthesized proteins, guiding translocation of proteins across membranes, disassembling oligomeric protein structures and facilitating degradation of some proteins [1]. HSP function as a main protective mechanism against harmful environmental factors, as their expression is rapidly upregulated in response to stressful stimuli (increased temperature, nutritional deficiency, oxidative stress, irradiation, etc.). In addition, it was demonstrated that HSP exert potent immunostimulatory properties: they were shown to interact with the specific cell surface receptors inducing the expression of proinflammatory mediators, Th1-inducing cytokines [2] and β-chemokines [3]. Because of high sequence homology displayed between microbial and endogenous HSP, immunological cross-reactivity to microbial HSP has been suggested as a possible cause of the development of autoimmune diseases, such as rheumatoid arthritis (RA) [4].

Rheumatoid arthritis is a chronic inflammatory disease characterized by chronic joint inflammation with infiltration of macrophages and activated T cells. Although a number of antigens as well as genetic and environmental factors were implicated in its pathogenesis, to this day the underlying pathogenic mechanisms of RA remain largely elusive. Experimental models of RA in use at present are numerous – the first one to be described was adjuvant arthritis (AA), which can be induced in susceptible rat strains by single injection of Freund's complete adjuvant (CFA), containing *Mycobacterium tuberculosis* [5]. This experimental model of RA can be induced in genetically susceptible rat strains, such as Dark Agouti (DA) [6, 7], Lewis [8], or Wistar [9]. The disease cannot be induced in genetically resistant rat strains, for example, Albino Oxford (AO) [10].

So far, the mycobacterial antigen that is solely responsible for AA induction was not identified, although there is a body of evidence that the immune response to HSP65 plays a significant role. First evidence of a role of HSP as antigens in inflammatory responses was obtained when it was demonstrated that AA could be triggered by a cross-reactive $CD4^+$ T-cell clone that recognized an epitope present on bacterial HSP65 as well as on cartilage proteoglycans [11]. However, administration of HSP65 to susceptible rat strains led to protection from disease induction that was mediated by T cells specific for HSP65 [12]. Mycobacterial HSP71 (mHSP71) belonging to a HSP70 gene family was also able to induce a suppression of AA, while not being arthritogenic itself [13]. The significance of the immune response to members of HSP70 family for the arthritis pathogenesis was also confirmed by finding of the elevated anti-HSP70 antibodies in sera of RA patients [14].

HSP47 (also known as colligin, gp46 and rheumatoid arthritis-related antigen, RA-A47) is a collagen-specific glycoprotein which resides in endoplasmic reticulum, and is involved in synthesis and assembly of collagen molecules as a molecular chaperone. The induction of HSP47 expression is prominent in the process of fibrosis irrespective of tissue or organ involved, which suggests its role in fibrotic processes by increasing synthesis and assembly of collagens [15]. A role of HSP47 in pathogenesis of RA was also suggested: it was shown that RA-A47 appeared on the cell surface concomitant with upregulation of metabolic factors related to cartilage destruction, and that this exposure of HSP47 on the cell surface is a factor that enables immune inflammatory reactions to start and result in autoimmune destruction [16].

This study addressed the immune response to HSP47 and mHSP71, as well as the level of HSP expression in joints of DA rats, susceptible, and AO, resistant, to the induction of AA. We were interested to determine whether the level of expression of HSP47 and/or HSP72 (rat homologue of mHSP71) in joints changed as a consequence of immunization with CFA and whether it was in correlation with the disease development. In addition, we aimed to determine if the antibodies specific for HSP47 and/or mHSP71 contributed to the susceptibility or the resistance to the induction of AA.

Materials and methods

Animals. A 6-month-old male inbred rats of two rat strains, DA ($n = 41$) and AO ($n = 27$), were used in experiments. DA rats were derived from our breeding colony at the Immunology Research Center 'Branislav Janković', Belgrade, while AO rats were obtained from the Military Medical Academy, Belgrade. Animals were housed in standard cages with free access to food pellets and tap water. All procedures involving animals and their care were approved by our Institutional Animal Care and Use Committee and followed principles described in the European Community's Council Directive of 24 November 1986 (86/609/EEC).

Antigens and antibodies. Recombinant human HSP47 (Colligin, Product # NSP-535), recombinant *M. tuberculosis* HSP71 (Product # SPP-885), recombinant rat HSP70 (HSP72, Product # SPP-758), mouse anti-HSP47 monoclonal antibody specific for both human and rat HSP47 (Product # SPA-470), mouse anti-mycobacterial HSP71 monoclonal antibody (Product # SPA-885) and mouse anti-HSP70 alkaline phosphatase conjugate monoclonal antibody (Product # SPA-810AP) were obtained from Stressgen Biotechnologies (Victoria, Canada). Anti-rat IgG-alkaline phosphatase and anti-mouse IgG-alkaline phosphatase were purchased from Sigma (St Louis, MO, USA). Recombinant human HSP47 and recombinant *M. tuberculosis* HSP71 were tested for their endotoxin content by quantitative Limulus amebocyte lysate (LAL) kinetic chromogenic assay (Reader: Tecan Sunrise, 405 nm, Charles River Endosafe, Charleston, SC, USA; Software: ENDOSCAN-V). Endotoxin contents of both proteins tested were found to be below limit of quantitation of the assay, i.e. <0.05 EU/μg of protein.

Induction and assessment of arthritis. In order to induce AA, a total of 50 rats of DA ($n = 32$) and AO ($n = 18$) strain were immunized with a single 0.05 ml intradermal injection of CFA (paraffin oil 8.5 ml and Arlacel A 1.5 ml, containing 6 mg/ml of *Mycobacterium bovis*, French 1173P2) in the basis of the tail. A total of nine animals of each strain were left non-immunized and served as a control.

Animals were daily scored for clinical signs of AA according to scale from 0 to 16, with each of four paws being scored from 0 to 4 as follows: 0 = no arthritic changes; 1 = oedema and/or arthritic nodules on one finger; 2 = oedema and/or arthritic nodules on two fingers; 3 = oedema and/or arthritic nodules on three fingers; 4 = oedema and/or arthritic nodules on all fingers [17].

Sera collection. Rats were bled by cardial puncture in light ether anaesthesia on 7, 21 and 35 days post-immunization (dpi). The blood was allowed to clot for 1 h at +4 °C. Sera were separated by two centrifugal cycles at 300 g for 15 min and stored at −20 °C until ELISA was performed. Individual groups of animals were killed on 7, 21 and 35 dpi, therefore sera obtained at different time points originated from different individual animals.

ELISA. Flat bottom 96-well plates (Nunc, Maxisorp, Roskilde, Denmark) were coated overnight with 100 μl per well of HSP47 or mHSP71 (2 μg/ml) in carbonate buffer pH 9.6, at 4 °C. After washing four times with 0.05% PBS-Tween-20, the plates were saturated with 200 μl per well of 1% BSA in PBS for 1.5 h, at room temperature. Following washing with 0.05%

PBS-Tween-20, the plates were inverted and tapped onto clean paper tissue until dry, followed by incubation with rats sera (diluted 1/50 in 1% BSA, 0.05% Tween-20 in PBS) and the positive control (mouse monoclonal anti-HSP47 or anti-mHSP71 antibody, 1 μg/ml in 1% BSA, 0.05% Tween-20 in PBS) for 1.5 h, at room temperature. After rewashing, the wells previously incubated with rat sera were incubated with anti-rat IgG-alkaline phosphatase, while the positive control wells were incubated with anti-mouse IgG-alkaline phosphatase for 1 h at room temperature. After washing, the presence of anti-HSP47 and anti-mHSP71 antibodies was detected by incubation with alkaline phosphatase substrate, *p*-nitrophenylphosphate (*p*-NPP, 1 mg/ml in 10 mM diethanolamine, 0.5 mM $MgCl_2$) for 30 min at room temperature. The optical density was measured photometrically at 405 nm. The number of sera samples per time point was 6 for AO and 8–12 for DA rat strain. The number of sera samples obtained from non-immunized animals was 9 for both DA and AO rats.

Preparation of rat joint homogenates protein extract. Wrist and ankle joints were wiped off with alcohol, removed and homogenized *in toto* in 5 ml per 1 g of joint weight of extraction buffer (5% SDS, 5% 2-mercaptoethanol, 20 mM Tris, 20 mM glycine, 2 mM Na_2EDTA, pH 6.8), on ice. Proteolysis was prevented by addition of protease inhibitors cocktail for mammalian tissues, P 8340 (104 mM AEBSF, 80 μM Aprotinin, 2 mM Leupeptin, 4 mM Bestatin, 1.5 mM Pepstatin, 1.4 mM E-64; Sigma) as well as 0.2 M phenylmethylsulphonyl fluoride (PMSF). Homogenized tissues were centrifuged at 22,000 g for 15 min, at 4 °C; the supernatants were filtered through 0.22 μm filters, aliquoted into criotubes and stored at −80 °C until use. The homogenates were prepared from the joints of both DA and AO animals collected on days 0, 7, 21 and 35 after the immunization, therefore eight experimental groups of extracts were prepared. A total of six to eight animals of each strain were killed per time point, their joints harvested, pooled together and extracted.

SDS-PAGE. Joint homogenates protein extracts as well as HSP47 and rat HSP72 positive control proteins were subjected to vertical SDS-PAGE according to the method of Laemmli [18] using 9% separating and 4% concentrating gel (0.75 mm width) on 'Mini Protean II' system (Bio-Rad Laboratories, Richmond, CA, USA). Gel polymerization was induced by ammonium persulphate (APS; Pharmacia, Uppsala, Sweden) and TEMED (Serva, Heidelberg, Germany), and the gels were blotted onto nitrocellulose membranes. Positive control proteins were loaded at 0.5 μg per lane, while joint homogenates protein extracts were loaded at 5 μg of total protein per lane.

Preliminary SDS-PAGE, followed with Coomassie Brilliant Blue staining, of HSP-positive controls, joint homogenates protein extracts and the molecular weight markers was performed in order to confirm the molecular weights of the control HSP, as well as the effectiveness of protein extraction from joints (results not shown). Once the required parameters were confirmed in preliminary experiments, the molecular weight markers were not loaded on gels in the immunoblotting experiments. An additional low-intensity protein band was noted in HSP47 preparation at a higher than 47 kDa molecular weight (close to the 94 kDa molecular weight standard), possibly originating from impurities or the residual dimeric HSP47.

Molecular weight protein standards were obtained from Bio-Rad Laboratories (phosphorylase B, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbon anhydrase, 30 kDa; soybean trypsin inhibitor, 21.1 kDa; α-lactoalbumin, 14.4 kDa).

Immunoblotting. Electrophoretically separated joint proteins and positive control proteins were electrotransferred to nitrocellulose paper NC 2 (pore size of 0.2 μm; Serva) as described elsewhere [19], using 1 mA/cm^2 electricity for 1 h 20 min at room temperature. Nitrocellulose membranes were incubated in blocking buffer containing 1.5% skim milk (Sigma) in PBS for 1 h at 37 °C, followed by washing with 0.05% Tween-20 in PBS and incubated with mouse monoclonal anti-HSP47 antibody (1 μg/ml in 1% BSA, 0.05% Tween-20 in PBS) for 1 h at room temperature. After washing, the membranes were incubated with the secondary antibody, anti-mouse IgG-alkaline phosphatase for 1 h at room temperature and stained by addition of the substrate [nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolylphosphate (BCIP); Sigma]. For detection of HSP72 nitrocellulose membranes were incubated with anti-HSP70 alkaline phosphatase-conjugated mouse monoclonal antibody. The reaction was stopped by washing the blots with the distilled water; blots were dried at room temperature and photographed.

Cell culture and BrdU staining. Inguinal lymph nodes draining the CFA injection site were isolated 21 days after immunization with CFA from both DA ($n = 10$) and AO rats ($n = 6$), and single-cell suspensions were prepared. The inguinal lymph node cells (LNC) isolated from naive animals ($n = 6$ from each strain) were used as control (0 dpi). The number of cells was adjusted to 2×10^6/ml, and 100 μl per well of the cell suspension was added to the microtitre plate. The cells were incubated for 24 h on 37 °C/5% CO_2 with 100 μl per well of 10 μg/ml hsp47 or 10 μg/ml mHSP71 (final concentration of HSP 5 μg/ml) in cRPMI (RPMI-1640/10% FCS/2 mM L-glutamine/200 μg/ml streptomycin/200 IU/ml penicillin). After the incubation period 10 μl of 2×10^{-4} M BrdU solution (prepared from the 3×10^{-2} M BrdU stock in DPBS) was added per well, and the plates were incubated for 18 h on 37 °C/5% CO_2. Staining with anti-BrdU-FITC and 7-AAD was performed according to the procedure supplied with the BrdU kit (Becton Dickinson, San

Jose, CA, USA). Anti-CD3-PE (Becton Dickinson) was used to differentiate between T cells and non-T cells. Samples (total of 2×10^4 flow cytometric events) were analysed on a FACScan flow cytometer (CELL QUEST software, Becton Dickinson).

Statistical analysis. All biometric calculations (mean, SD, SE) were performed using statistical packages SPSS 10. In order to determine significant differences between the independent groups one-factor ANOVA was used followed by Fisher's *post hoc* test. Strain differences were tested by means of *t*-test. Differences are regarded as statistically significant if $P < 0.05$. Results are presented as mean + SE.

Results

HSP47 and HSP72 expression in rat joints

The expression of HSP47 and HSP72 in rat joints during the course of AA was analysed by Western blot. In DA rats immunized with CFA intense foot swelling was accompanied by frequent arthritic nodules in all rats, while no clinical signs were observed in AO animals. Clinical signs in DA rats appeared on 11 dpi, the maximal clinical signs were reached on 23 dpi, while the disease slowly regressed towards 35 dpi (Fig. 1A).

In the control extract prepared from the DA animal that was not subjected to immunization (0 dpi) the expression of HSP47 was barely detectable. Immunization with CFA also did not induce the increase in HSP47 expression on day 7 following immunization, before the clinical signs have appeared. However, a marked increase in HSP47 expression in joints of DA rats which expressed severe clinical signs of AA was noted on 21 dpi, as well as on day 35 after the arthritis induction (Fig. 1B).

HSP47 was not detectable in joints originating from non-immunized AO rats (0 dpi), suggesting that this protein was probably expressed at fairly low levels under the physiological conditions, which could not be detected due to limitations of the method applied (Fig. 1C). Contrary to the results obtained for DA rat strain, the expression of HSP47 in joints of AO rats was not affected by immunization with CFA, as HSP47 remained undetected regardless of the dpi on which the joints were collected and extracted (Fig. 1C).

HSP72 has been detected in both DA (Fig. 1D) and AO (Fig. 1E) joint extracts of non-immunized animals, and the level of its expression did not change following immunization with CFA (7, 21 and 35 dpi). In AO rats, however, the level of HSP72 expression was fairly low, and the protein bands barely visible.

Humoral immune response to HSP47 and mHSP71

The levels of anti-HSP47 and anti-mHSP71 antibodies in DA and AO rats' sera collected on days 0, 7, 21 and 35 after immunization with CFA were determined by ELISA method. Sera of non-immunized rats of both strains (0 dpi) showed a certain level of HSP47 immunoreactivity (Fig. 2A). It appeared that DA rats sera contained slightly higher levels of natural anti-HSP47 antibody (0 dpi) in comparison with AO sera, although this difference was not significant. The immunization of DA rats with CFA induced an increase in the anti-HSP47 antibody level in sera obtained on the peak of disease, on 21 dpi. Although not statistically significant, slight increase in anti-HSP47 level was also observed in sera of AO rats obtained on day 21 following immunization with CFA. On 21 dpi, the level of anti-HSP47 antibody was statistically significantly higher in DA rat sera in comparison with the levels found in sera obtained from AO rats immunized with CFA.

Anti-mHSP71 antibodies have been detected in sera of non-immunized (0 dpi) DA and AO rats, and were of a similar level (Fig. 2B). The induction of AA in DA rats did not influence the level of anti-mHSP71 antibodies, while the immunization with CFA increased serum levels of anti-mHSP71 antibodies in AO rats on 21 and 35 dpi. However, on 7 dpi, the serum level of these antibodies was significantly higher in DA rats compared with AO rats.

Lymphocyte proliferation and apoptosis in response to HSP47 and mHSP71

There were no strain differences in basal proliferation (cultured in RPMI only) of $CD3^+$ and $CD3^-$ LNC obtained from non-immunized rats (0 dpi, Fig. 3A,B). Immunization with CFA significantly increased and decreased basal proliferation of $CD3^+$ LNC from AO and DA rat strain, respectively. The greater percentage of both $CD3^+$ and $CD3^-$ LNC of non-immunized AO rats underwent apoptosis in RPMI culture, when compared with corresponding cells from DA rats (Fig. 3C,D). In contrast, there were no strain differences in LNC apoptosis after immunization with CFA (21 dpi), and apoptosis of $CD3^-$ cells was significantly decreased in both strains relative to 0 dpi (Fig. 3D). In addition, small changes attributable to immunization with CFA were observed in percentage of apoptotic $CD3^+$ LNC of AO and DA rats, but in opposite directions (Fig. 3C).

The present study aimed to investigate whether the *in vitro* incubation with HSP47 or mHSP71 of inguinal LNC isolated from DA and AO rats on day 21 after immunization with CFA would have significant effect on $CD3^+$ and $CD3^-$ cells proliferation and apoptosis. In DA rats, HSP47 and mHSP71 did not stimulate $CD3^+$ LNC proliferation over the basal level (RPMI), but decreased it in AO rats (Fig. 4A). However, proliferation of $CD3^+$ LNC in RPMI, as well as after incubation with HSP47

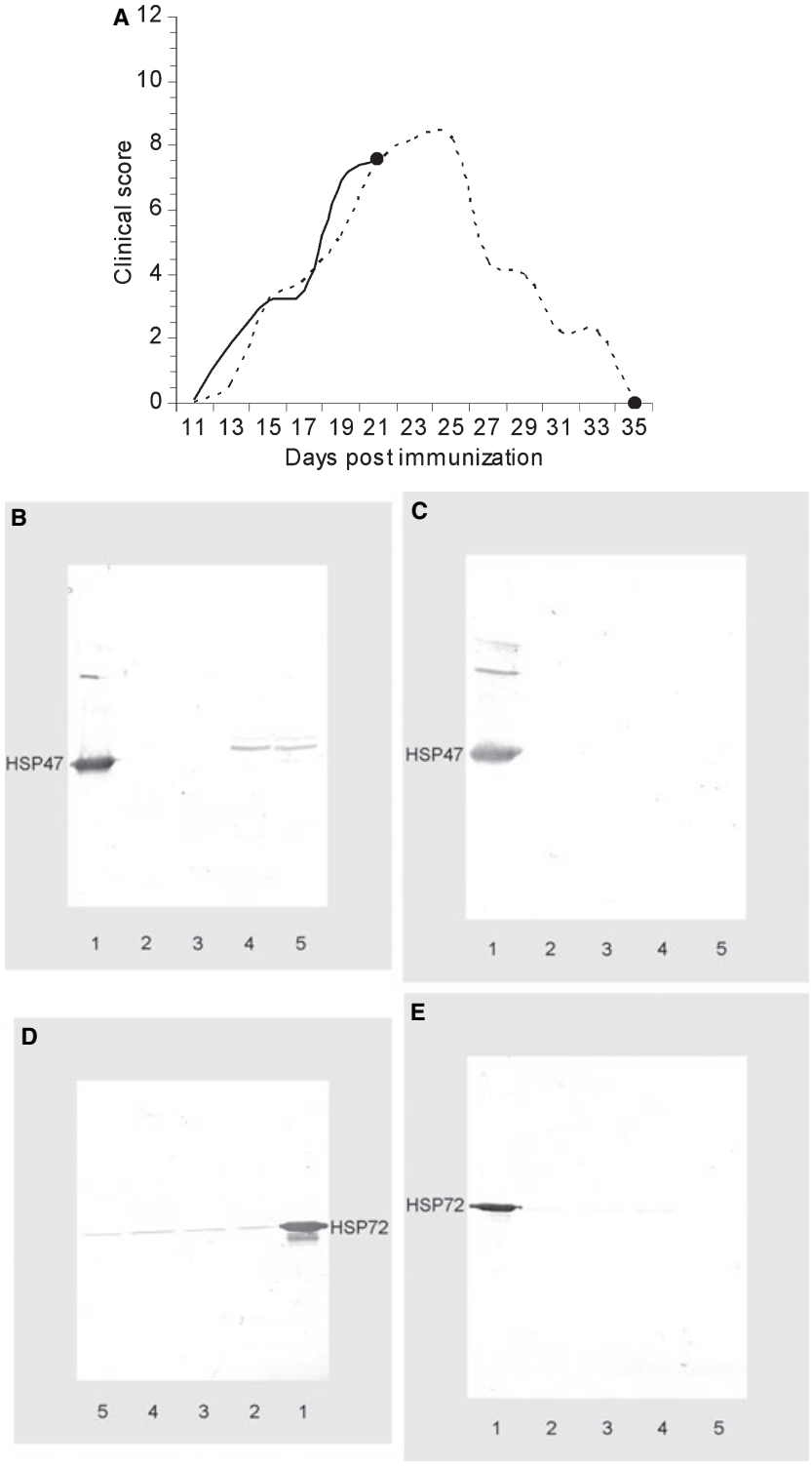

Figure 1 Clinical signs of adjuvant arthritis in Dark Agouti (DA) rats till 35 dpi (dashed line) and 21 dpi (full line; A), and Western blot analysis of joint homogenates extracts from DA (B and D) and Albino Oxford rats (C and E). Protein extracts were prepared on 0, 7, 21 and 35 dpi, and probed with anti-HSP47 (B and C) and anti-HSP70 (D and E) antibody. Lanes assignment: 1 = positive control; 2 = dpi 0; 3 = dpi 7; 4 = dpi 21; 5 = dpi 35.

and mHSP71, was significantly increased in AO strain when compared with DA strain. With respect to $CD3^-$ LNC, HSP47 and mHSP71 reduced proliferation in both AO and DA strain (Fig. 4B). Besides, strain differences in $CD3^-$ cell proliferation capacity were observed after *in vitro* incubation with RPMI and with mHSP71, showing higher percentage of proliferating cells in AO strain in comparison with DA strain.

HSP47 did not affect apoptosis of $CD3^+$ LNC, while mHSP71 increased it in DA, but not in AO strain (Fig. 4C). The percentage of apoptotic $CD3^+$ LNC was not significantly different between the two rat strains

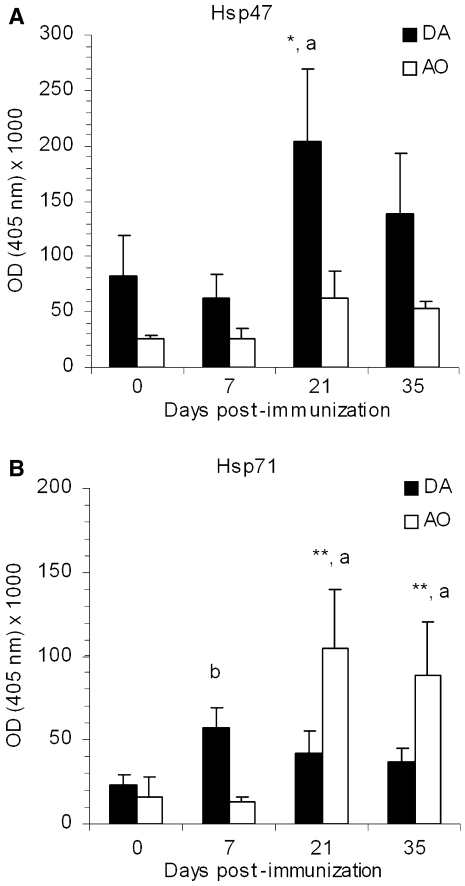

Figure 2 Strain differences in serum anti-HSP47 (A) and anti-mHSP71 (B) antibody levels in Dark Agouti (DA) and Albino Oxford (AO) rats. Sera were obtained before immunization with complete Freund's adjuvant (0 dpi) and on 7, 21 and 35 dpi. The number of sera samples was 6–12 per group. The results are expressed as $OD_{405} \times 1000$ + SE. Statistically significant differences: $^{*}P < 0.05$; $^{**}P < 0.01$, versus 0 and 7 dpi; and $^{a}P < 0.05$; $^{b}P < 0.01$, DA versus AO.

following the *in vitro* incubation with HSP47 or with mHSP71. With respect to $CD3^{-}$ LNC, HSP47 decreased and mHSP71 increased percentage of apoptotic cells in DA strain (Fig. 4D), while both stress proteins showed no effect on the apoptosis of $CD3^{-}$ LNC obtained from AO rats immunized with CFA. However, a significantly lower percentage of apoptotic cells after incubation with mHSP71 was detected in AO rats compared with DA rats (Fig. 4D). No strain differences in apoptosis of both $CD3^{+}$ and $CD3^{-}$ LNC in RPMI were observed (Fig. 4C,D).

Discussion

Markedly increased level of expression of HSP47 in joints of DA rats which developed the clinical signs of AA, particularly at the time of disease peak, most likely reflected the tissue damage progress. HSP47 is a collagen-binding stress protein that acts as a collagen-specific molecular chaperone during the biosynthesis and secretion of procollagen in the living cell. It is a membrane glycoprotein that resides in endoplasmic reticulum and has the ability to bind to native collagen type I, III and IV, as well as to gelatine [20, 21]. Earlier research results have pointed out to HSP47 overexpression in various pulmonary fibrotic diseases characterized by abnormally increased synthesis and deposition of collagen molecules [22]. There is ample evidence that the increased synthesis of HSP47 is involved with the abundant production of type I procollagen [23]. The expression of HSP47 always correlates with that of collagens, while the cells in which collagen synthesis is not detected do not synthesize detectable amounts of HSP47, as well [24]. As the tissue destruction in joints affected by arthritis is a stressful signal to cells, elevated production of stress proteins in joints is to be expected. In addition, with the progression of clinical symptoms of arthritis the increased production of collagen molecules and the onset of fibrosis is also taking place, therefore requiring the increased expression of HSP47 essential for procollagen molecules processing.

Our finding that the levels of HSP72 expression in DA and AO rats joint extracts were not different before and after immunization with CFA suggests that strain difference in the susceptibility to AA induction in these two rat strains is independent on HSP72 expression in the tissue specifically affected by the disease. Conversely, increased expression of HSP70 has been detected in synovial tissue from patients with RA [25–27].

Proinflammatory cytokines, such as tumour necrosis factor (TNF)-α, interferon (IFN)-γ and interleukin (IL)-6 downregulate gene expression of HSP47 in chondrocytes [28]. However, despite their effect on the HSP47 gene level, these cytokines have also caused surface expression of HSP47 and, associated with these phenomena, upregulation of mRNA for metabolic factors, such as inducible NO synthase (iNOS) and matrix metalloproteinase (MMP)-9. The appearance of HSP47 on the cell surface following downregulation of its gene expression was attributed to apoptosis [16]. The change in localization of HSP47 molecule to the cell surface is regarded as the possible mechanism for its recognition as an autoantigen during RA.

An increase in anti-HSP47 antibody level simultaneously with the protein upregulation in diseased joints observed in DA rat strain is in agreement with the previous findings that HSP47 at some point translocates to the cell surface to be recognized by the immune cells under stressful conditions. As the anti-HSP47 antibody level was significantly increased at the peak of disease, this opens a question whether HSP47-specific antibodies could be a part of perpetuating or exaggerating mechanisms in the pathophysiology of AA. The immunoreactivity towards the chondrocyte-derived HSP47 was

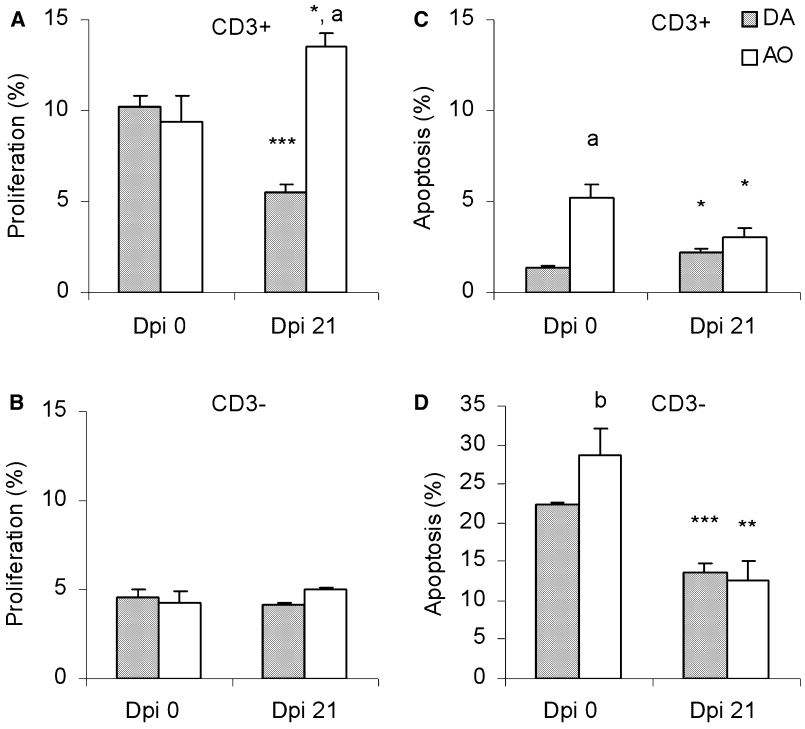

Figure 3 Strain differences in $CD3^+$ and $CD3^-$ inguinal lymph node cell (LNC) proliferation (A and B) and apoptosis (C and D) in RPMI. Inguinal LNC were obtained from Dark Agouti (DA) and Albino Oxford (AO) animals immunized with complete Freund's adjuvant on 21 dpi. Inguinal LNC from non-immunized rats (0 dpi) of both strains served as a control. The number of samples was 6–10 per group. Statistically significant differences: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, versus corresponding control (0 dpi); and $^aP < 0.001$, and $^bP < 0.01$, AO versus DA.

reported earlier in sera of osteoarthritis patients [29], making way for the hypothesis that an autoimmune reaction against chondrocytes is a possible mechanism in the pathophysiology of osteoarthritis. In addition, it was suggested that the antibody titre against the HSP47-like protein derived from the chondrocytes could be a possible marker for the prognosis of the joint pathology. As HSP47 was not detected in joints of AO rats, small increase in sera anti-HSP47 antibody level most likely reflected non-specific activation of B lymphocytes following immunization with CFA. Interestingly, we have observed that particular levels of natural anti-HSP47 antibodies were detectable in sera of normal, healthy, non-immunized rats of both DA and AO strains. HSP47 is a chaperon expressed in endoplasmic reticulum of vertebrates and is not found in prokaryotes; therefore there is no possibility that the presence of antibodies to HSP47 is due to immune cross-reactivity between the hosts HSP47 and its microbial counterpart. Similarly, occurrence of natural autoantibodies for another HSP, gp96 that resides within endoplasmic reticulum has been explained as a consequence of enhanced cell surface expression of gp96 during stressful conditions, or alternatively as a result of cell death during normal development in thymus [30]. The pre-existing response may serve to downregulate stronger pathological autoimmune response to HSP, as it was observed that vaccination with HSP preparations did not lead to an enhancement of the anti-HSP antibody titre [30]. A potential physiological role of both natural and acquired anti-HSP47 antibodies is yet to be determined in future research.

The rise in anti-mHSP71 antibody levels in sera of AO rats following immunization with CFA advocates for a possible protective role of these antibodies in AA. It has been shown earlier that pre-immunization with mHSP71 ameliorated clinical signs of AA, and arthritis induced with collagen II or synthetic adjuvant avridin [13]. Similarly, a protective role of antibodies specific for another mycobacterial stress protein, HSP65, has been reported in Wistar rat strain subjected to AA induction [31]. Anti-HSP65 antibodies passively transferred resistance to AA induction from resistant to susceptible rat strain [32]. In addition, in pristan-induced arthritis increased levels of anti-HSP65 antibodies were detected only in sera of mice that did not develop clinical signs of arthritis [33].

It was suggested earlier that the adjuvant oil exerts its major proarthritogenic activity in the lymph nodes, including the ones draining the injection site, rather than directly in the joints [34]. Similarly, Svelander *et al.* [35] have pointed out to the increased lymphocyte proliferation in the inguinal lymph nodes following immunization with incomplete Freund's adjuvant. We were interested to determine whether strain differences existed in proliferative response of inguinal LNC at the peak of AA in susceptible and resistant rat strains that would account for their susceptibility/resistance to the disease. The finding that immunization with CFA increased the $CD3^+$ LNC proliferation in AO rats, and decreased it in DA rats indicated that these two strains differentially respond to antigen stimulation *in vivo*. Vigorous apoptotic response of both $CD3^+$ and $CD3^-$ LNC in AO rats can

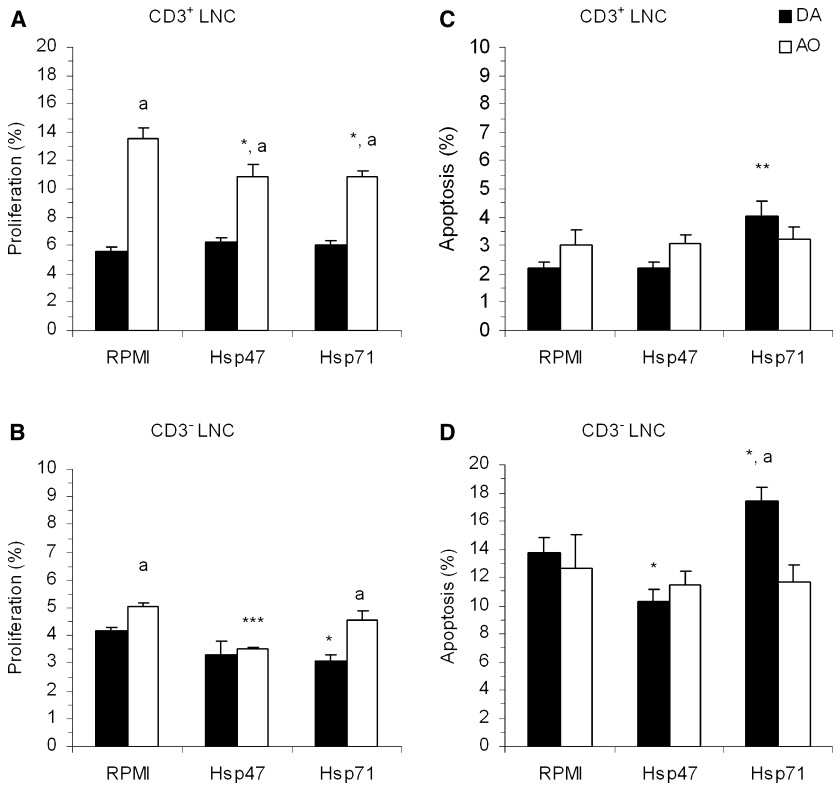

Figure 4 Strain differences in $CD3^+$ and $CD3^-$ inguinal lymph node cell (LNC) proliferation (A and B) and apoptosis (C and D) following the *in vitro* treatment with 5 μg/ml of HSP47 or 5 μg/ml of mHSP71 in RPMI. Inguinal LNC were obtained from Dark Agouti (DA) and Albino Oxford (AO) animals immunized with complete Freund's adjuvant on 21 dpi. The number of samples was 6–10 per group. Statistically significant differences: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, versus RPMI; and $^aP < 0.01$, AO versus DA.

to a certain extent explain resistance of this strain to the induction of autoimmune diseases, having in mind that apoptosis is one of the mechanisms for elimination of self-reactive and potentially autoimmune lymphocytes [36]. It was shown earlier using the same arthritis model that a lack of apoptosis in ankle joints may contribute to disease progression in arthritis-susceptible rats [37].

In addition, this study aimed to determine if the immunization with CFA primed LNC specific for HSP47 or for mHSP71 that would lead to their proliferation in response to *in vitro* exposure to these molecules. There seems to be a suppressive effect of the HSP47 molecule on T- and B-cell proliferation in AO rat strain, but the method used in this study could not determine the T-cell or the B-cell phenotype or their antigen specificity and therefore could not pinpoint the T- and B-cell population being most affected. One can speculate the overall immune response – including the potentially proarthritogenic response – is being suppressed as a consequence of stimulation with HSP47 in AO strain. This effect can clearly be attributed to HSP47, as the immunization with CFA has shown to cause the significant increase in T-cell proliferation in AO rats 21 days after immunization.

To the best of our knowledge this is the first data reported to suggest T-cell immune response to HSP47 may be of importance for the pathogenesis of arthritis, although further research in this area is certainly necessary.

Our finding that mHSP71 induced a decrease in proliferation and an increase in apoptosis of $CD3^-$ LNC from DA rats compared to AO rats is in line with low levels of anti-mHSP71 antibodies found in DA rats sera after immunization with CFA. Furthermore, higher proliferative response to mHSP71 of $CD3^+$ LNC from AO rats relative to DA rats is compatible with the protective role of mHSP71 in AA, as reported earlier [13]. It has been shown recently that vaccination with DNA-encoding HSP70 inhibited development of AA in Lewis rats by inducing shift in arthritogenic T-cell phenotype from Th1 towards Th2/Th3 [38].

In conclusion, our work points out to possible roles of HSP47 in AA development: the increase of HSP47 expression in arthritic joints as well as the increase of serum anti-HSP47 antibody levels seem to be concomitant with the tissue damage progression. In addition, this stress protein reduces proliferation of T and B cells in AA-resistant rat strain, leading to a hypothesis that HSP47 participates in AA control. The immune response to mHSP71 in AA-resistant rat strain reflected in elevated antibody production in combination with decreased B-cell proliferative response and increased apoptosis in AA-susceptible strain, are all indicative of a protective role for anti-mHSP71 antibodies in AA.

Having chosen one HSP of a mycobacterial origin and the other one being endogenous, we started off with the hypothesis that the immune response to mHSP71 in a resistant rat strain will be proven to be a protective one,

the protein itself being non-arthritogenic. This was shown in AO strain; however, the *in vitro* stimulation with mHSP71 in DA rats confirmed and even potentiated the inadequate response of these rats to the *in vivo* mycobacterial antigen stimulation. On the other hand, the immune response to an endogenous stress protein, not present in CFA preparation but associated with AA, has proven to follow the arthritic signs development in susceptible rats, as expected. However, the ability of the endogenous HSP47 to clearly suppress both T- and B-cell immune response in AA-resistant strain while not being expressed in joints of these rats, points out to the presence of immune cells specific to this protein that might be activated by the CFA immunization and which are capable of keeping the immune response – including the potentially arthritogenic one – under control.

Authors contributions

TM drafted the manuscript and was involved in study design, experimental procedures performance and data analysis. MD, VKJ and SS were involved in study planning and design, experimental procedures performance, data analysis and manuscript preparation. MD performed statistical analysis of the data. VV and KM were involved in experimental procedures performance and data analysis. DK performed the analysis on FACScan flow cytometer and provided pertinent data interpretation. All authors approved the final manuscript.

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