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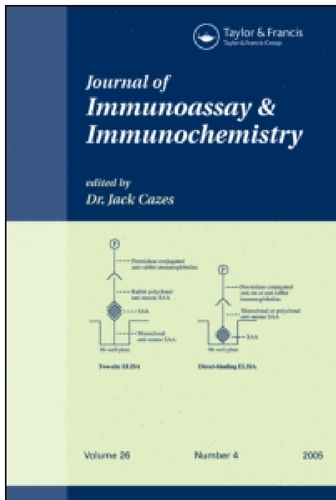

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Humoral Response Against *Mycobacterium bovis* Hsp65 Derived Fragments in Children and Young People with Various Disorders

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Abstract: Using Western blotting, we investigated IgG antibodies against *Mycobacterium bovis* heat shock protein 65 (MB-Hsp65) fragments produced by cleavage with cyanogen bromide (CNBr) in 10 healthy controls, 11 patients with juvenile idiopathic arthritis (JIA), and 10 children with various diseases before haematopoietic stem cell transplantation (HSCT). CNBr cleaved MB-Hsp65 to three larger fragments: P1-163, P191-285, and P290-534. Sera of JIA patients and those before HSCT reacted with individual MB-Hsp65 fragments P1-163 and P290-534 significantly more frequently when compared with healthy controls. These results suggested that the key B-cell epitopes of MB-Hsp65 might be located on the aforementioned sequences.

Keywords: Antibodies; Haematopoietic stem cell transplantation; Juvenile idiopathic arthritis; Protein cleavage; Western blotting

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INTRODUCTION

Increasing evidence shows that proteins of HSP60 family (heat shock proteins with molecular weight of 60 kDa) are involved in pathogenesis of different disorders, e.g., autoimmune or cardiovascular diseases. Hsp65, a member of HSP60 family, is isolated from *Mycobacterium* species. Sequence comparison of Hsp65 from different *Mycobacterium* strains showed that the protein sequence of *Mycobacterium bovis* (MB) is identical to that of *M. tuberculosis*, and very similar to that of *M. leprae*, the pathogens that cause tuberculosis and tuberculoid leprosy, respectively.[1]

MB-Hsp65 derived from Bacille Calmette-Guérin (BCG) vacccine was identified as the immunodominant antigen during mycobacterial diseases and vaccination.[2] Hsp65 is of critical significance in rat adjuvant arthritis (AA), an experimental model of autoimmune arthritis that is induced by intracutaneous injection with heat-killed mycobacteria in complete Freund's adjuvant (CFA).[3] Both arthritogenic (A2b) and protective (A2c) T cell clones obtained from rats with AA recognized the 180–188 sequence (TFGLQLELT) of mycobacterial Hsp65.[4] A2b seems to cross-react with a structure derived from cartilage proteoglycan, suggesting that targeting of inflammation to the joints might be due to cross-reactivity between aa 180–188 of Hsp65 and a self component in the cartilage.[5] Hsp65 also plays a crucial role in the T cell regulatory events involved in both the induction of AA and protection against AA.[6]

In humans, a high proportion of children affected by juvenile idiopathic arthritis (JIA) showed both antibody and T lymphocyte responses to Hsp65 and to two related peptides: the 180–188 sequence of Hsp65 and a partially homologous peptide of the cartilage proteoglycan link protein. The titer of circulating antibodies was generally higher in patients with clinically active disease.[7]

Our studies showed elevated proliferative response of peripheral blood mononuclear cells (PBMC) to MB-Hsp65 and P180-188 MB-Hsp65 derived peptide in JIA patients, mainly in patients with polyarthritis with established disease lasting more than 2 years.[8] Concerning humoral response, we reported significant increases of IgG antibodies raised against MB-Hsp65 and its epitope 180–188 in patients with JIA when compared with healthy controls.[9]

In other studies, we also demonstrated a significantly high proliferative response to MB-Hsp65 in a cohort of immunocompromised paediatric patients suffering from malignant and non-malignant diseases with anamnestic and/or actual infection when compared to a cohort of patients without infection, as well as healthy individuals.[10] Next, we showed that anti- MB-Hsp65 IgG antibodies were detected in sera of these patients before haematopoietic stem cell transplantation

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(HSCT).[11,12] However, there was no significant difference in IgG antibody levels between patients and healthy controls.

Although diseases such as adjuvant arthritis (AA), rheumatoid arthritis (RA), and juvenile idiopathic arthritis (JIA) are generally categorized as predominantly T-cell mediated, there is increasing information pointing toward the role of antibodies in the disease process. Ulmansky et al.[13,14] reported that resistance of AA is due to the presence of natural, as well as acquired anti-Hsp65 antibodies and the protective epitopes are B cell epitopes with nonconserved aa sequences found on the outer surface of molecule Hsp65. Thus, more investigations on detection of antibodies against MB-Hsp65 derived epitopes in humans are needed.

The aim of this study was to determine a humoral reactivity of paediatric and young patients with various malignant and non-malignant diseases before HSCT and patients with JIA against MB-Hsp65 derived fragments generated by cyanogen bromide (CNBr) digestion. Thus, we were able to determine whether there were qualitative and quantitative differences in the epitopes recognized by the anti-Hsp65 antibodies in different patients' cohorts when compared to healthy controls.

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EXPERIMENTAL

Patients

Ten patients undergoing HSCT for various malignant and non-malignant diseases, eleven patients with JIA, and ten healthy controls were included in the study.

The first tested cohort consisted of 10 paediatric patients (8 males, 2 females; age range 1–17 years, mean 9.4, median 8) treated with allogeneic HSCT in the Bone Marrow Transplant Unit in the Department of Paediatric Haematology and Oncology at University Hospital Motol in Prague. The underlying diseases in transplanted patients were acute lymphoblastic leukaemia (ALL, n = 2), acute myeloid leukaemia (AML, n = 3), severe aplastic anaemia (SAA, n = 1), Wiskott-Aldrich syndrome (WAS, n = 2), and myelodysplastic syndrome (MDS, n = 2). Patients' sera were collected before conditioning (range D-65-D-4). Patients received peripheral blood stem cell (PBSC, n = 4) or bone marrow (BM, n = 6) grafts from unrelated donors (n = 9) and/or HLA identical siblings (n = 1).

The further cohort consisted of eleven patients (8 males, 3 females; age range 12–30 years, mean 18.6, median 18) from the Outpatient Department of Rheumatology at University Hospital Motol in Prague with definite JIA lasting more than two years (range 2–25 years). The underlying diseases, using the Idiopathic Arthritides of Childhood Classification criteria, were 3 oligoarthritis, 6 polyarthritis and 2 systemic

arthritis[15]. All patients were rheumatoid factor (RF) negative. A total cohort involved 1 antinuclear antibody (ANA) positive and 10 ANA negative; 3 human leukocyte antigen (HLA) B27 positive and 8 HLA B27 negative patients. Patients met the standard American College of Rheumatology (ACR) criteria for disease activity measures and were divided into two groups depending on disease activity 1) complete or near remission with or without on-going treatment (n = 4) and 2) active disease (n = 7).

Sera of ten age-matched, healthy controls (4 males, 6 females; age range 2–30 years, mean 16, median 16) were also tested.

All of the tested individuals were given BCG vaccination during the period from the 4th day to the 6th week after birth. Children with negative tuberculin skin test were re-vaccinated at the age of two, similarly at the age of 11 or 12 years.

Local ethics committee approval and informed consents were obtained for all individuals involved in the study. Sera were aliquoted and stored at −80°C until used.

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Cleavage of MB-Hsp65 by CNBr

MB-Hsp65 fragments were produced by digesting 1 mg of purified lyophilised MB-Hsp65 (Lionex, Braunschweig, Germany) with 600 μL of 0.5 M CNBr/70% TFA (Trifluoroacetic Acid), (Sigma Biosciences, St Louis, MO, USA).[16] The cleavage was performed at room temperature in the dark for 24 hours. After the cleavage, the sample was lyophilised by evaporation in a Speed-Vac for 4 hours. The mixture of fragments was tested using Tricine-Sodium DodecylSulfate-Polyacrylamide Gel Electrophoresis and Western blotting.

Identification of MB-Hsp65 Derived Fragments by Tricine-Sodium DodecylSulfate-Polyacrylamide Gel Electrophoresis (Tricine SDSPAGE)

Tricine SDSPAGE was performed by a modification of the method described by Schagger and von Jagow,[17] with an 18% separating gel and a 5% stacking gel. The sample containing either MB-Hsp65 or Hsp65 derived fragments was diluted in sample buffer, heated at 95°C for 4 minutes and electrophoresed for 15 minutes at 50 V, followed by 90 minutes at 140 V at concentration 2 μg/lane for MB-Hsp65 and 5 μg/lane for its fragments using Mini-Protean 3 Cell (Bio-Rad, CA, USA). The gels were stained with 0.25% Coomassie Brilliant Blue solution (Bio-Rad, CA, USA) or by Silver staining kit for proteins (Roti^{R}-Black P, Carl Roth GmbH + Co., Karlsruhe, Germany).

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Western Blotting (WB)

By using WB, each serum sample was tested at least twice to confirm the results.

MB-Hsp65 fragments separated by Tricine SDSPAGE were transferred to Polyvinylidene difluoride (Immobilon-P^{SQ}) membranes (Millipore, MA, USA) in a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, CA, USA) at 100 V, 350 mA for 90 minutes at room temperature (RT). The efficiency of the transfer was confirmed by staining the membranes with 0.1% amido black solution (Sigma Biosciences, St Louis, MO, USA). The membranes were rinsed with PBS-0.1% Tween 20 (PBST) (Bio-Rad, CA, USA) and blocked with 5% non-fat dry milk (Bio-Rad, CA, USA) in PBS for 2 h at RT. Patients' sera were diluted 1:50 in blocking buffer and incubated with the membranes for 2 h at RT with gentle agitation. After washing, horseradish peroxidase conjugated goat anti-human IgG antibody (172–1050, Bio-Rad, CA, USA) diluted 1:3,000 in blocking buffer was incubated with the membranes for 90 minutes at RT.

Anti-mycobacterial Hsp65 monoclonal antibody (SPA-882, Stressgen, Victoria, Canada), together with goat anti-mouse IgG horseradish peroxidase conjugated antibody (170–6516, Bio-Rad, CA, USA) was used as relevant control for WB assay.

Simultaneously, omission of the incubation with sera or monoclonal antibody had always been performed to confirm the specificity of the assays.

After washing, bound anti-Hsp65 antibodies were detected by using an Amplified Opti-4CN Substrate Kit (Bio-Rad, CA, USA) according to the manufacturer's instructions.

Analysis of the results was accomplished by capturing strip images, locating bands using prestained molecular weight marker (Precision Plus Protein standards 10–250 kDa, Bio-Rad, CA, USA), measuring the reflectance density (DR1, after subtraction of background value) of bands with AlphaEaseFC Stand Alone software (Alpha Innotech, San Francisco, USA). To increase the reproducibility of measurements, we calculated the ratio of antibody concentrations (DR2) in each experiment: DR2 = DR1 of antibodies against MB-Hsp65 epitope/DR1 of antibody against MB-Hsp65.

Statistical Analysis

A two-tailed Student's t-test was used for the statistical analysis. P values of less than 0.05 were regarded as significant.

RESULTS AND DISCUSSION

Using tool "peptide cutter" (www.expasy.com), which predicts potential protease and cleavage sites and sites cleaved by chemicals in a given protein sequence, we predicted that CNBr cleaving proteins at methionine should digest MB-Hsp65 into four larger fragments: Fragment No.1 (Fr-1): amino acid position (aa) 1–163, molecular weight (Mw) 17.1 kDa; fragment No.2 (Fr-2): aa 164–190, Mw 2.9 kDa; fragment No.3 (Fr-3): aa 191–285, Mw 10.4 kDa; fragment No.4 (Fr-4): aa 290–534, Mw 25.3 kDa.

The staining, either with Coomassie Brilliant Blue solution or by the much more sensitive Silver staining kit for proteins, revealed three major bands corresponding to the prediction: Fr-1 (17.1 kDa), Fr-3 (10.4 kDa), Fr-4 (25.3 kDa). A number of partial digestion products were also present in the mixture (Figure 1).

Representative WB results, which are shown in Figure 2 and Table 1, include all detailed results.

MB-Hsp65 reacted with anti-mycobacterial Hsp65 monoclonal antibody (DR1 = 118). However, none of the fragments derived from MB-Hsp65 showed reactivity with anti-mycobacterial Hsp65 monoclonal antibody. The background of the assay was 4 (DR1 = 4). Similarly, as we reported previously, IgG antibodies against a whole molecule MB-Hsp65 were detected in all tested sera, including paediatric patients with various malignant and non-malignant diseases before HSCT, JIA patients, and healthy controls.[11] IgG antibodies against P1-163 epitope (Fr-1, 17.1 kDa) were obviously detected in 7/10 (70%) of patients before HSCT, 7/11 (63.6%) of JIA patients, and none of the 10 healthy controls. Positivity of antibodies against P191-285 epitope (Fr-3, 10.4 kDa) occurred in 7/10 (70%) of patients before HSCT, 7/11 (63.6%) of JIA patients, and 4/10 (40%) of healthy controls. The presence of antibodies against P290-534 epitope (Fr-4, 25.3 kDa) was observed in 9/10 (90%) of patients before HSCT, 8/11 (72.7%) of JIA patients and 5/10 (50%) of healthy controls.

While IgG anti-Hsp65 antibody levels showed no significant differences between these small studied cohorts, significantly higher levels of antibodies against MB-Hsp65 epitopes were observed in patients before HSCT and JIA patients, when compared with healthy controls (Table 2). Comparing DR1 (9.6 vs 4.8, $p = 0.014$; 12.2 vs 6.6, $p = 0.009$) and DR2 (0.24 vs 0.09, $p = 0.022$; 0.3 vs 0.13, $p = 0.003$) values, significantly elevated antibodies against P1-163 (Fr-1, 17.1 kDa) and P290-534 (Fr-4, 25.3 kDa) epitopes were found in a cohort of patients before HSCT. Significantly increased DR1 (9.0 vs 4.8, $p = 0.018$; 10.9 vs 6.6, $p = 0.05$) and DR2 (0.19 vs 0.09, $p = 0.006$; 0.21 vs 0.13, $p = 0.04$) values of antibodies against P1–163 (Fr-1,

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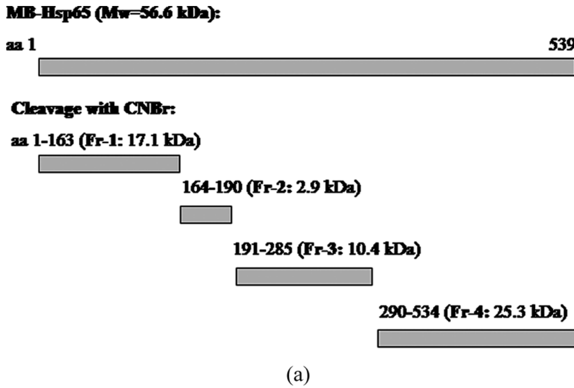

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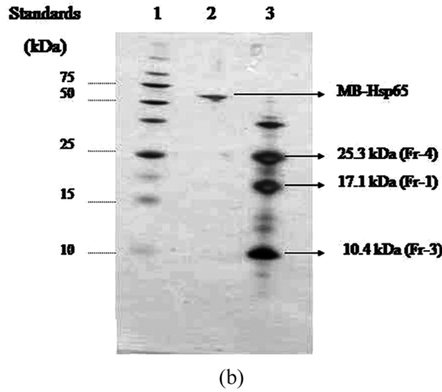

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Figure 1. Prediction and result of MB-Hsp65 cleavage with CNBr. (1-A) According to the tool "peptide cutter" (www.expasy.com), CNBr cleaving proteins at methionine should digest MB-Hsp65 to four larger fragments: Fragment No.1 (Fr-1): amino acid position (aa) 1–163, molecular weight (Mw) 17.1 kDa; fragment No.2 (Fr-2): aa 164–190, Mw 2.9 kDa; fragment No.3 (Fr-3): aa 191–285, Mw 10.4 kDa; fragment No.4 (Fr-4): aa 290–534, Mw 25.3 kDa. (1-B) MB-Hsp65 (0.5 µg/lane) and MB-Hsp65 fragments gained after cleavage with CNBr (5 µg/lane) were run on 18% Tricine SDSPAGE and stained by Silver staining kit for proteins. Lane 1: Precision Plus Protein standards – BIO-RAD (250, 150, 100, 75, 50, 37, 25, 20, 15, 10 kDa). Lane 2: MB-Hsp65. Lane 3: the separation of three main larger fragments of MB-Hsp65 after cleavage with CNBr.

17.1 kDa) and P290–534 (Fr-4, 25.3 kDa) epitopes were also detected in a cohort of JIA patients.

The levels of antibodies against P191-285 epitope (Fr-3, 10.4 kDa) were higher in both cohorts of patients (patients before HSCT 10.0 vs 5.1; $p = 0.05$ and JIA 8.5 vs 5.1; $p = 0.08$) when compared with healthy

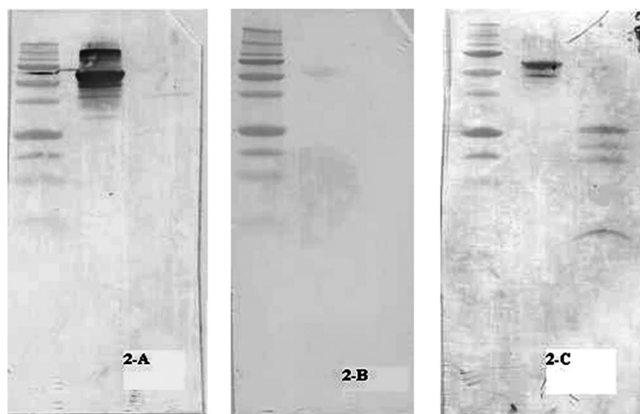

Figure 2. Representative WB results. MB-Hsp65 (2 μg/lane) (lanes 2) and a mixture of MB-Hsp65 derived fragments (5 μg/lane) (lanes 3) were run on 18% Tricine SDSPAGE and blotted against anti-mycobacterial Hsp65 monoclonal antibody (SPA-882, Stressgen, Victoria, Canada)(MAb) diluted 1:1000 (2-A) or sera in dilution 1:50 in a blocking buffer (5% non-fat dry milk in PBS) (2-C). MB-Hsp65 reacted with anti-mycobacterial Hsp65 monoclonal antibody. The reflectance density (DR1, after subtraction of background value) was 118. However monoclonal antibody did not react with any fragments derived from MB-Hsp65 (DR1 = 5). The background of the assay (DR1 value obtained from the lanes in which serum samples were substituted with 5% non-fat dry milk in PBS) was 4 (2-B). Lanes 1: Precision Plus Protein standards – BIO-RAD (250, 150, 100, 75, 50, 37, 25, 20, 15, 10 kDa). Lanes 2: MB-Hsp65 reacted with either monoclonal antibody or patient's serum. Lanes 3: the positivity of antibodies against three fragments derived from MB-Hsp65 in serum of patient with WAS.

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controls where the difference nearly reached the statistical significance. However, no statistical difference between the studied groups was observed when the ratio of antibodies against P191-285 epitope (Fr-3, 10.4 kDa) and antibodies against MB-Hsp65 was calculated (patients before HSCT 0.22 vs 0.18; $p = 0.35$ and JIA 0.21 vs 0.18; $p = 0.40$).

In this study, we examined humoral response of paediatric and young patients with different malignant and non-malignant diseases before HSCT and patients with JIA to MB-Hsp65, as well as MB-Hsp65 derived fragments obtained after cleavage by CNBr.

Similarly, as we reported previously, anti-Hsp65 IgG antibodies were detected in all tested sera.[11] The antibody levels against the whole molecule of MB-Hsp65 did not differ between either patients' or healthy control' groups. However, regarding antibodies against various MB-Hsp65 fragments, we could observe the qualitative and quantitative difference between the studied cohorts. Concerning healthy controls, the humoral

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Table 1. Detailed patients' clinical characteristics and positivity of IgG antibodies against MB-Hsp65 and MB-Hsp65 derived fragments

Patients pre-HSCT							
UPN	Diagnosis	Age/Sex	Clinical characteristics (Disease duration in months; infection around the time of serum sampling)	Anti-Hsp65	Anti-Fr-1 (17.1 kDa)	Anti-Fr-3 (10.4 kDa)	Anti-Fr-4 (25.3 kDa)
SCT 1	AML-CR2	17/M	15 m; *E.coli* sepsis 4 months before	+	+	+	+
SCT 2	AML-CR1	13/M	5 m; w/o	+	+/−	+	+
SCT 3	AML-CR3	7/M	22 m; lung aspergilossis 1 month before	+	+	−	+
SCT 4	MDS	17/F	7 m; lung aspergilossis and *S. epidermidis* sepsis 1 month before	+	+	+/−	+
SCT 5	MDS	16/M	6 m; w/o	+	+	+	+
SCT 6	SAA	5/M	36 m; fungal pneumoniae (*Rhizopus*) 3 months before	+	−	+	+
SCT 7	WAS	4/M	36 m; without complication	+	+/−	+	+/−
SCT 8	WAS	5/M	1 m; recurrent abscess (*S. aureus*)	+	+	+	+
SCT 9	ALL-CR1	1/F	5 m; recurrent infections (*Acinetobacter, Pseudomonas aeruginosa, Burgholderia*) 2 months before	+	+	+	+
SCT 10	ALL-CR2	9/M	47 m; w/o	+	+	−	+
Number of sera reactive with MB-Hsp65 and MB-Hsp65 fragments				10/10	7/10	7/10	9/10

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Table 1. Continued

JIA patients

UPN	JIA subtype	Age/Sex	Clinical characteristics (Laboratory parameters; disease duration in years; disease activity and therapy at the time of serum sampling)	Anti-Hsp65	Anti- Fr-1 (17.1 kDa)	Anti-Fr-3 (10.4 kDa)	Anti-Fr-4 (25.3 kDa)
JIA 1	Systemic	18/M	RF (–); ANA (–); HLA B27 (–); 3 y; active disease; DMARD	+	+	+	+/−
JIA 2	Systemic	12/M	RF (–); ANA (–); HLA B27 (–); 3 y; remission; C/DMARD	+	+	+	+
JIA 3	Polyarthritis	22/F	RF (–); ANA (–); HLA B27 (–); 5 y; remission; C/DMARD	+	+	+	−
JIA 4	Polyarthritis	13/M	RF (–); ANA (–); HLA B27 (–); 3 y; remission; DMARD	+	+/−	+/−	+
JIA 5	Polyarthritis	24/F	RF (–); ANA (–); HLA B27 (+); 2 y; active disease; DMARD	+	+	+	+

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JIA 6	Polyarthritis	30/M	RF (–); ANA (–); HLA B27 (–); 25 y; active disease; C/DMARD	+	+	+	+
JIA 7	Polyarthritis	22/M	RF (–); ANA (–); HLA B27 (–); 7 y; active disease; C/DMARD/NSAID	+	+	−	+
JIA 8	Polyarthritis	21/M	RF (–); ANA (–); HLA B27 (+); 14 y; active disease; C/DMARD	+	+	−	+
JIA 9	Oligoarthritis	13/F	RF (–); ANA (–); HLA B27 (–); 2 y; active disease; C/DMARD	+	−	+	−
JIA 10	Oligoarthritis	18/M	RF (–); ANA (+); HLA B27 (–); 13 y; remission; without therapy	+	+/−	+	+
JIA 11	Oligoarthritis	12/M	RF (–); ANA (–); HLA B27 (+); 2 y; active disease; C/DMARD	+	+/−	+/−	+
Number of sera reactive with MB-Hsp65 and MB-Hsp65 fragments				11/11	7/11	7/11	8/11

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Table 1. Continued

Healthy controls					
UDN	Age/Sex	Anti-Hsp65	Anti- Fr-1 (17.1 kDa)	Anti-Fr-3 (10.4 kDa)	Anti-Fr-4 (25.3 kDa)
HD 1	14/F	+	+/−	−	+
HD 2	14/M	+	+/−	−	+
HD 3	17/M	+	+/−	−	+/−
HD 4	2/F	+	−	+	−
HD 5	19/M	+	−	+	+
HD 6	18/F	+	+/−	−	+
HD 7	18/F	+	+/−	−	−
HD 8	13/M	+	−	+	−
HD 9	15/F	+	−	+	+
HD 10	30/F	+	+/−	+/−	+/−
Number of sera reactive with MB-Hsp65 and MB-Hsp65 fragments		10/10	0/10	4/10	5/10

ALL: acute lymphoblastic leukaemia; ANA: antinuclear antibody; AML: acute myeloid leukaemia; C: corticosteroids; CR: complete remission; DMARDs: disease modifying antirheumatics; *E. coli: Escherichia coli*; F: female; HD: healthy donor; HLA: human leukocyte antigen; M: male; m: month; MDS: myelodysplastic syndrome; NSAIDs: non-steroid antirheumatics; RF: rheumatoid factor; SAA: severe aplastic anaemia; *S. epidermidis*: Staphylococcus epidermidis; UDN: unique donor number; UPN: unique patient number; WAS: Wiskott-Aldrich syndrome; y: year.

Table 2. Statistical analysis of DR1 (densitometric values) and DR2

	MB-Hsp65	Fr-1 (17.1 kDa)	Fr-3 (10.4 kDa)	Fr-4 (25.3 kDa)
DR1 values (mean and range)				
Patients before HSCT	45.1	9.6	10.0	12.2
	23–62	0–20	0–17	6–20
JIA patients	48.7	9.0	8.5	10.9
	15–86	0–18	0–17	0–25
Healthy controls	46.7	4.8	5.1	6.6
	9–96	0–12	0–23	0–18
P values (DR1) – T test				
Patients before HSCT × controls	0.421	0.014	0.05	0.009
JIA patients × controls	0.411	0.018	0.08	0.05

	Fr-1 (17.1 kDa)	Fr-3 (10.4 kDa)	Fr-4 (25.3 kDa)
DR2 values (mean and range)			
Patients before HSCT	0.24	0.22	0.3
	0–0.74	0–0.43	0.13–0.57
JIA patients	0.19	0.21	0.21
	0–0.33	0–0.41	0–0.40
Healthy controls	0.09	0.18	0.13
	0–0.21	0–0.89	0–0.26
P values (DR2) – T test			
Patients before HSCT × controls	0.022	0.35	0.003
JIA patients × controls	0.006	0.40	0.04

JIA: juvenile idiopathic arthritis; HSCT: haematopoietic stem cell transplant.

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reactivity against MB-Hsp65 derived fragments differs on a case by case basis despite the fact that all individuals were vaccinated with the same BCG tuberculosis vaccine during childhood. We observed evincible positivity of antibodies against Fr-4 in 3 cases, against Fr-3 in 2 cases and against both of them in 2 cases. That might suggest that the immune system of each individual would react to different epitopes of MB BCG Hsp65 immunodominant antigen.

The sera, either of JIA patients or those before HSCT, reacted with individual MB-Hsp65 fragments more frequently (Table 1). Seven patients altogether showed reactivity against all of the fragments, 5 patients had antibodies against Fr-1 and Fr-4, 3 patients against Fr-3 and Fr-4, 2 patients against Fr-1 and Fr-3, and 2 patients against Fr-3 or Fr-4.

Significantly elevated levels of IgG antibodies to Fr-1 and Fr-4 were observed in cohorts of pre-HSCT and JIA patients when compared with

healthy controls. Fr-2, which contains well-known T-cell epitope P180-188, was not detected in Tricine SDSPAGE, probably due to its low molecular weight.[18]

Heat shock proteins (HSPs) are highly conserved during evolution, which has resulted in extensive amino acid sequence identities between mammalian and microbial HSPs. In spite of this homology, microbial HSPs have been found to be strong immunogens. Immune reactivity against different members of HSP families, most frequently Hsp60, Hsp70, and Hsp90, accompanies many infectious diseases.[19] An increased humoral response against individual Hsp65 derived fragments in a cohort of patients before HSCT might be expected, since immunocompromised paediatric patients suffering from different malignant and non-malignant diseases had been frequently affected by infection including various bacteria and fungi. In our tested cohort, seven out of ten patients suffered from anamnestic or actual infection such as *Escherichia coli* sepsis, fungal pneumonia, or recurrent infections caused by *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Acinetobacter*, and *Burgholderia* species (Table 1). Most patients were also at present colonized with various bacteria.

In the previous study, we examined peripheral blood mononuclear cell (PBMC) responses to HSPs in relation to infection inpaediatric patients with various lympho-haemopoietic malignancies, as well as non-malignant disorders subjected to HSCT. We demonstrated significantly high proliferative response to recombinant human Hsp60, as well as to MB-Hsp65 in a cohort of patients with anamnestic and/or actual infection when compared to a cohort of patients without infection and healthy individuals. Strong PBMC cell 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.[10]

As well, we observed that anti-HSP antibodies might be produced even after HSCT in relation to infection depending on aetiological agents. We demonstrated de novo humoral response to HSPs in a cohort of patients with actual infection caused by *Klebsiella pneumoniae* (anti-Hsp60, anti-Hsp65, and anti-Hsp70), *Pseudomonas aeruginosa* (anti-Hsp60, anti-Hsp70) and *Aspergillus fumigatus* (anti-Hsp65).[12]

An increased humoral response against individual Hsp65 derived fragments in a cohort of patients with JIA might be explained by several means. Infection is one of the well-known mechanisms responsible for the induction of autoimmune inflammation, after which HSP synthesis is greatly increased as a response to a variety of stressful stimuli. Due to a very high interspecies sequence homology between HSP the immune response to microbial HSP may cross-react with self-HSP. Furthermore, the cross reactivity between HSP and other self-proteins might intensify the autoimmune process. Hence, enhanced levels of IgG antibodies to MB-Hsp65 derived fragments detected in sera of patients with JIA might reflect anamnestic infection responsible for

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the onset of the autoimmunity, as well as the response against highly expressed Hsp60 in site of inflammation due to a sequential homology between human Hsp60 and MB-Hsp65. It was reported that synovial lining cells of patients with JIA show an increased expression of endogenously produced Hsp60[20] and IgG antibodies to human Hsp60 can be detected in both serum and synovial fluid from patients with JIA.[21] Enhanced proliferative response of PBMC derived from JIA patients after stimulation with MB-Hsp65 was also reported.[7,8,22]

Recent studies have shown that antibodies produced during the course of AA protect against subsequently induced AA in Lewis rats.[14,23] It has been speculated that antibodies against HSP suppress inflammation by inhibiting the proinflammatory effect of the HSP on the innate immune system. The protective antibodies induce secretion of both IL-10 and TNF-α by murine and human mononuclear cells, but the increase in IL-10 secretion was 1.6- to 6.8-fold greater than that of TNF-α. Thus, the increase in IL-10 secretion in the inflammatory site can skew the local cytokine profile from an inflammatory to an anti-inflammatory response and, thus, explain the mechanism of protection against inflammation by these antibodies.[14]

Regarding patients with rheumatoid arthritis (RA), it is conceivable that a subsets of anti-mycobacterial Hsp65 antibodies might be involved in the regulation of acute RA. Several studies have shown the beneficial effect of transient depletion of B cells in RA patients and highlighted the contribution of these cells to the disease propagation by production of pathogenic antibodies, by secretion of cytokines, and by serving as antigen presenting cells (APCs).[24–26]

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CONCLUSION

Our results suggest that, in the case of JIA patients or paediatric patients with various malignant and non-malignant diseases subjected to HSCT, the key B-cell epitopes of MB-Hsp65 might be located on the sequences P1-163 and P290-534 of MB-Hsp65. Further studies examining the role of antibodies against MB-Hsp65 B-cell epitopes in different patients' cohorts are required to clarify the roles of these antibodies in pathogenesis of the diseases.

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