

Reactivity of γ/δ T Cells to Human 60-kd Heat-Shock Protein and Their Cytotoxicity to Aortic Endothelial Cells in Takayasu Arteritis

Sunil Kumar Chauhan,¹ Mahavir Singh,² and Soniya Nityanand¹

Objective. Increased numbers of circulating γ/δ T cells with a restricted T cell receptor repertoire, as well as colocalization of the expression of heat-shock protein Hsp60/65 and γ/δ T cells in the arterial lesions of patients with Takayasu arteritis (TA), indicate that γ/δ T cells may react to Hsp60 and cause damage to the arterial endothelium. In this study we investigated the proliferative responses of γ/δ T cells to human Hsp60 and their cytotoxicity to human aortic endothelial cells (ECs) in patients with TA.

Methods. Blood samples were obtained from 12 patients with TA, 8 patients with systemic lupus erythematosus (SLE) (as disease controls), and 10 healthy control subjects. Proliferative responses of circulating γ/δ T cells to human Hsp60 were detected by flow cytometry–based bromodeoxyuridine incorporation assay. Cytotoxicity of the γ/δ T cells to human aortic ECs was analyzed by colorimetric lactate dehydrogenase release assay.

Results. The γ/δ T cells of 11 of 12 patients with TA exhibited reactivity to Hsp60, whereas none of the γ/δ T cells from patients with SLE or healthy controls showed reactivity (both $P < 0.001$). The mean \pm SD proliferative response of γ/δ T cells in patients with TA was $21.4 \pm 11.3\%$, compared with $4.2 \pm 1.2\%$ in patients with SLE and $4.01 \pm 1.82\%$ in healthy controls (both

$P < 0.001$). In addition, compared with the control groups, the γ/δ T cells of patients with TA had increased spontaneous cytotoxicity to aortic ECs ($22.1 \pm 15.0\%$ versus $9.6 \pm 2.13\%$ in SLE patients and $8.1 \pm 4.7\%$ in healthy controls; both $P < 0.005$), which was further enhanced following stimulation of γ/δ T cells with Hsp60. The cytotoxicity of the γ/δ T cells was significantly inhibited by treatment of these cells with concanamycin A and anti-Fas ligand–blocking antibodies.

Conclusion. The results show that γ/δ T cells in patients with TA are reactive to Hsp60 and exhibit cytotoxicity to aortic ECs, suggesting a key role of Hsp60 and γ/δ T cells in the pathogenesis of TA.

Takayasu arteritis (TA) is a chronic granulomatous arteritis characterized by intimal thickening, fibrosis, and stenosis as well as aneurysm of the large elastic arteries, predominantly the aorta and its major branches. Its etiology is largely unknown, but most of the available data suggest that immune-mediated dysfunction of the arterial endothelium is a primary event in the pathogenesis of the disease (1–3).

We previously have demonstrated an increased number of circulating activated γ/δ T cells with a restricted T cell receptor (TCR) repertoire in patients with TA (4). Similarly, Seko et al observed the predominance of TCR-restricted γ/δ T cells in the arterial lesions of patients with TA (5). These findings indicate an antigen-driven activation and involvement of these cells in the pathogenesis of the disease. Since a major human heat-shock protein (HSP) antigenic target of γ/δ T cells is Hsp60 (6), and Hsp60/65 is highly expressed in the arterial lesions of patients with TA (7), it appears that γ/δ T cells recognize HSPs or some homologous arterial antigens and transmigrate from the circulation to the vascular wall, thus causing arterial damage that culminates in different clinical manifestations of the disease.

In a recent study (8) we detected the presence of

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anti-endothelial cell (anti-EC) antibodies that were predominantly directed against the 60–65-kd aortic EC antigen, which was suggestive of endothelial Hsp60. These findings indicate that γ/δ T cells may react to this 60–65-kd EC antigen and lead to damage of the endothelium in TA. However, there are no data available on the reactivity of γ/δ T cells to Hsp60 and their cytotoxicity to arterial endothelium in TA. We therefore undertook this study to investigate the proliferative responses of γ/δ T cells to human Hsp60 and their cytotoxicity to human aortic ECs, as well as the mechanism of their cytotoxicity, in patients with TA.

PATIENTS AND METHODS

Subjects. Twelve patients with TA (9 female, 3 male, mean \pm SD age 28.6 ± 7.7 years, range 20–44 years), 10 age- and sex-matched healthy control subjects, and 8 patients with systemic lupus erythematosus (SLE) as disease control subjects (6 female, 2 male, mean \pm SD age 30.5 ± 10.8 years, range 16–49 years) were included in the present study, which was approved by the institutional ethics committee. All of the patients with TA fulfilled the American College of Rheumatology 1990 criteria (9) and had angiographically proven disease.

Hsp60 stimulation and purification of γ/δ T cells.

Fifteen microliters of heparinized venous blood was obtained from each subject, and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density gradient centrifugation. The PBMCs were suspended at a concentration of 2×10^6 cells/ml and stimulated with $10 \mu\text{g/ml}$ of recombinant human Hsp60 for 7 days under standard tissue culture conditions, as described previously (10). The recombinant Hsp60 was expressed in *Escherichia coli* and was completely identical to human Hsp60, without any expressed fusion partner (Lionex Diagnostics & Therapeutics, Braunschweig, Germany).

An immunomagnetic method was used to isolate γ/δ T cells (γ/δ T Cells Isolation Kit; Miltenyi Biotech, Bergisch Gladbach, Germany) (11). Briefly, PBMCs were labeled with hapten-modified anti- γ/δ TCR antibodies and anti-hapten microbeads, and positive selection of cells was then carried out with magnetic columns. The efficiency of positive selection was evaluated by flow cytometric analysis. The purified γ/δ T cells were used immediately in cytotoxicity assays.

Assay of γ/δ T cell proliferation. The proliferative response of Hsp60-stimulated γ/δ T cells was studied by bromodeoxyuridine (BrdU) incorporation assay, as described previously (10). Briefly, PBMCs stimulated with Hsp60 in the same manner as described above were pulsed with $30 \mu\text{g/ml}$ of BrdU for the last 24 hours of culture, and the incorporation of BrdU into the DNA of the proliferating cells was measured by staining the cells with anti-BrdU–fluorescein isothiocyanate and anti- γ/δ TCR–phycoerythrin monoclonal antibodies (Becton Dickinson, Mountain View, CA). The stained cells were analyzed by flow cytometry using CellQuest software (Becton Dickinson). The cutoff value for defining a proliferative re-

sponse was calculated as the mean \pm 2 SD of the BrdU-positive T cell response in healthy control subjects.

Assay of γ/δ T cell-mediated cytotoxicity to aortic ECs. Normal human aortic ECs (Cambrex Bioscience, Walkersville, MD) at passages 4–6 (5×10^3 cells/well) and unstimulated or Hsp60-stimulated γ/δ T cells (5×10^4 cells/well) were plated in a 96-well culture plate and incubated for 4 hours at 37°C . The cytotoxicity of γ/δ T cells to aortic ECs was analyzed by colorimetric lactate dehydrogenase (LDH) release assay using the Cytotox-96 kit (Promega, Madison, WI), with results expressed as the percentage of cytotoxicity, calculated as $([\text{optical density [OD] of experimental LDH release} - \text{OD of } \gamma/\delta \text{ T cell spontaneous LDH release} - \text{OD of EC spontaneous LDH release}] / [\text{OD of EC maximum LDH release} - \text{OD of EC spontaneous LDH release}]) \times 100$.

To evaluate the cytotoxic mechanism of γ/δ T cells, perforin and Fas ligand (FasL) inhibition assays were carried out. The γ/δ T cells were incubated with concanamycin A (CMA) and/or anti-FasL–blocking antibodies, as described by Kataoka et al (12).

Statistical analysis. Analyses of data were carried out using the Mann-Whitney U test for comparison of mean values, and Fisher's exact test for analysis of frequency. Results are expressed as the mean \pm SD. *P* values less than or equal to 0.05, by 2-tailed test, were considered significant.

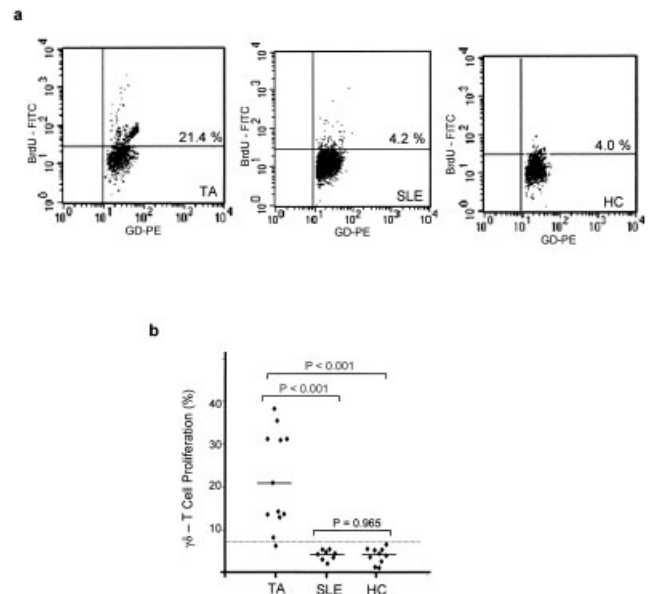


Figure 1. Representative flow cytometric dot plots (a) and scatter plot (b), showing the proliferative responses of γ/δ T cells to human Hsp60 in patients with Takayasu arteritis (TA), patients with systemic lupus erythematosus (SLE), and healthy control subjects (HC). Each dot in the scatter plot (b) represents data from an individual subject, and solid horizontal lines indicate the mean value for each group. The broken horizontal line represents the cutoff limit (mean \pm 2 SD of the control group) for defining a positive response. BrdU-FITC = anti-bromodeoxyuridine–fluorescein isothiocyanate monoclonal antibody; GD-PE = anti- γ/δ T cell receptor–phycoerythrin monoclonal antibody.

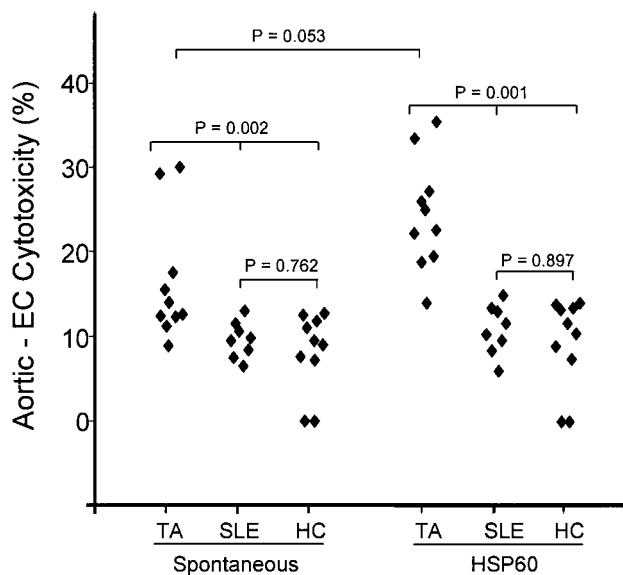


Figure 2. Scatter plot showing the cytotoxicity of unstimulated (spontaneous) and human Hsp60-stimulated γ/δ T cells to aortic endothelial cells (ECs) in patients with Takayasu arteritis (TA), patients with systemic lupus erythematosus (SLE), and healthy control subjects (HC). Each dot represents data from an individual subject.

RESULTS

Proliferative response of γ/δ T cells to Hsp60.

The γ/δ T cells in 11 (92%) of 12 patients with TA, compared with none of the patients with SLE (0 of 8) and none of the healthy control subjects (0 of 10) (both $P < 0.001$ versus patients with TA), exhibited proliferative responses to Hsp60. The proliferative response of γ/δ T cells in patients with TA was a mean \pm SD $21.4 \pm 11.3\%$, compared with $4.2 \pm 1.2\%$ in patients with SLE and $4.01 \pm 1.82\%$ in healthy control subjects (both $P < 0.001$) (Figures 1a and b).

Cytotoxicity of γ/δ T cells to aortic ECs. The γ/δ T cells of patients with TA compared with patients with SLE and healthy control subjects had a higher spontaneous cytotoxicity to aortic ECs (mean \pm SD $22.1 \pm 15.0\%$ versus $9.6 \pm 2.13\%$ in SLE patients and $8.1 \pm 4.7\%$ in healthy controls; both $P < 0.005$). Furthermore, this cytotoxicity of the γ/δ T cells in patients with TA was enhanced following stimulation of the cells with Hsp60 ($29.5 \pm 13.2\%$ versus $22.1 \pm 15.0\%$ in unstimulated cells; $P = 0.053$), but there was no difference in the cytotoxicity to aortic ECs between Hsp60-stimulated and unstimulated γ/δ T cells in patients with SLE ($10.9 \pm 2.9\%$ versus $9.6 \pm 2.13\%$; $P > 0.30$) and in healthy control subjects ($9.3 \pm 5.3\%$ versus $8.1 \pm 4.7\%$; $P > 0.30$) (Figure 2).

The cytotoxicity of Hsp60-stimulated γ/δ T cells in patients with TA was inhibited by treatment of these cells with CMA (mean \pm SD $9.5 \pm 2.2\%$; $P = 0.001$ versus untreated cells) or anti-FasL ($16.9 \pm 5.3\%$; $P = 0.003$ versus untreated cells). Combination treatment with CMA and anti-FasL also inhibited the cytotoxicity of Hsp60-stimulated γ/δ T cells in patients with TA ($5.5 \pm 2.8\%$; $P = 0.001$ versus untreated cells). In contrast, the cytotoxicity of Hsp60-stimulated γ/δ T cells that were left untreated was $29.5 \pm 13.2\%$ (Figure 3).

DISCUSSION

We have demonstrated that γ/δ T cells present in patients with TA are reactive to human Hsp60, and they possess spontaneous cytotoxicity to aortic ECs. Moreover, stimulation of γ/δ T cells with Hsp60 leads to further enhancement of their cytotoxic potential.

Recently we found that human Hsp60 induced a proliferative response of peripheral blood T cells in patients with TA; in addition, we observed the proliferation of a subset of double-negative (CD4⁻, CD8⁻) T cells (10). The results of our present study confirm that these double-negative T cells are Hsp60-reactive γ/δ T cells. Colocalization of Hsp60/65 expression and γ/δ T cells in arterial lesions as well as a restricted TCR gene usage of infiltrating and circulating γ/δ T cells in patients with TA further suggest that there is an Hsp60-driven expansion and infiltration of these cells in arterial lesions (4,5,7).

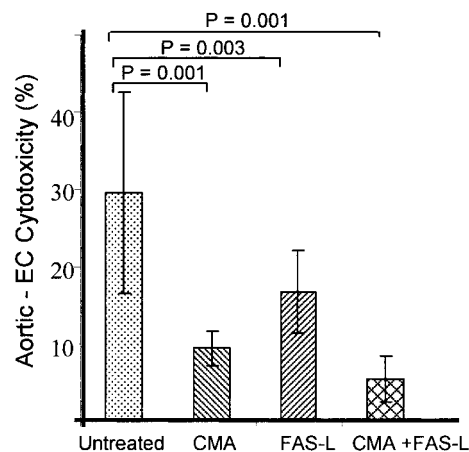


Figure 3. Results of inhibition studies, showing the cytotoxicity of untreated γ/δ T cells to aortic endothelial cells (ECs) compared with that of γ/δ T cells treated with concanamycin A (CMA), anti-Fas ligand (FAS-L)-blocking antibody, or CMA plus anti-FasL antibody in patients with Takayasu arteritis. Values are the mean \pm SD.

Similarly, γ/δ T cells present in patients with Behçet's disease have been reported to exhibit proliferative responses to various peptides of human Hsp60 (13), suggesting that Hsp60 may be an important stimulus responsible for *in vivo* stimulation and expansion of γ/δ T cells. In SLE patients, who comprised the disease control group in the present study, a previous study (14) found an increased frequency of γ/δ T cells (14), whereas we observed no proliferative response of γ/δ T cells against Hsp60 in patients with SLE. This suggests that the reactivity of γ/δ T cells to Hsp60 is specific to TA, since it was not found in either the SLE disease controls or healthy individuals. However, in addition to Hsp60/65, the vascular lesions of TA also show expression of stress-induced ligands, such as class I major histocompatibility complex (MHC) chain-related A (MICA), which is another antigenic target for γ/δ T cells (15). Thus, it is likely that MICA-reactive γ/δ T cells may also play a role in the vascular endothelial damage in TA.

It has been reported that γ/δ T cells recognize peptide fragments in the context of class I or class II MHC molecules or bacterial lipid antigens, occurring directly via CD1 molecules, and that γ/δ T cells are cytotoxic in nature and cause cytolysis of target cells via perforin- and Fas-mediated pathways (16). To evaluate the pathogenic relevance of these cells in TA, we investigated both their spontaneous and their Hsp60-induced cytotoxicity to aortic ECs, which is the cell type that is specifically targeted in the disease. It was observed that γ/δ T cells exhibited spontaneous cytotoxicity to aortic ECs, and that this cytotoxic potential of the cells was further enhanced following their stimulation with Hsp60.

We also carried out perforin and FasL inhibition studies to delineate the mechanisms involved in the cytolysis of aortic ECs by γ/δ T cells. The inhibition of either perforin or FasL each significantly reduced the cytotoxic potential of γ/δ T cells, while inhibition of both perforin and FasL concomitantly blocked the cytotoxic activity of these cells completely. Thus, the results of our inhibition studies showed involvement of both the perforin- and Fas-mediated pathways in the cytotoxicity of γ/δ T cells to aortic ECs. Previous histologic studies that showed a predominant infiltration of perforin-secreting γ/δ T cells and other killer cells in the arterial lesions of patients with TA lend support to our study findings (7). EC cytotoxicity of γ/δ T cells stimulated with mycobacterial lysate has also been reported in patients with scleroderma (17).

Our observations of the reactivity of γ/δ T cells to Hsp60 and their cytotoxicity to aortic ECs in TA suggest that Hsp60 may be a putative antigen involved in the activation and expansion of γ/δ T cells, which, in turn, may cause arterial damage attributable to the cytotoxicity of γ/δ T cells to ECs, which is mediated through both the perforin and Fas pathways. However, further studies are required to define the causes of Hsp60 expression in the vascular tissue of patients with TA and to gain a better understanding of the pathogenesis of the disease.

AUTHOR CONTRIBUTIONS

Dr. Nityanand had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Nityanand.

Acquisition of data. Chauhan.

Analysis and interpretation of data. Chauhan, Nityanand.

Manuscript preparation. Chauhan, Singh, Nityanand.

Statistical analysis. Chauhan, Nityanand.

Acquisition of Hsp60. Singh.

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Clinical Images: Earth, moon, and stars in a patient with gouty arthritis



The patient, a 55-year-old man who was undergoing a course of antituberculosis treatment, abruptly developed severe pain and swelling in the right knee and both ankles. Joint aspiration revealed thick, white material, which was confirmed by polarizing microscopy to be monosodium urate monohydrate crystals. The aggregate of crystals (right panels) resembled the painting “Starry Night” by Vincent van Gogh and the NASA photograph “Earth–Moon Conjunction” (courtesy NASA/JPL-Caltech) (left panels).

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