Heat Shock Treatment of Tumor Lysate-Pulsed Dendritic Cells Enhances Their Capacity to Elicit Antitumor T Cell Responses against Medullary Thyroid Carcinoma

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Background: In vitro and in vivo studies have shown that dendritic cells (DCs) can stimulate antitumor T cell responses against medullary thyroid carcinoma (MTC). However, despite promising results in selected cases, the clinical efficacy of DC immunotherapy in patients with MTC has been limited. Recently, it has been demonstrated in mice that heat shock enhances the capacity of bone-marrow-derived DCs to stimulate antigen-specific T cells. The aim of our investigations was to evaluate whether heat shock also increases the capacity of human monocyte-derived DCs to stimulate antitumor T cell responses against MTC tumor cells.

Methods: DCs from six patients with metastatic MTC were pulsed with tumor lysate derived from allogeneic MTC tumor cells and were heat shocked for 12 h at 40°C or kept at 37°C. Thereafter, the DCs were matured and cocultured with T cells. Finally, the cytotoxic activity of T cells against MTC tumor cells was measured in vitro.

Results: In all patient samples, cytotoxic T cell responses against MTC tumor cells could be induced. Notably, heat-shocked DCs were more potent stimulators of cytotoxic T cell responses than control DCs, with T cells stimulated with heat-shocked DCs displaying a significantly increased cytotoxic activity against MTC tumor cells as compared with T cells stimulated with control DCs. In none of the experiments was a cytotoxic T cell response against unrelated pancreatic tumor cells (PANC-1) observed, using both control and heat-shocked DCs.

Conclusions: Our study shows that heat-shocking DCs may be a valuable strategy to increase the immunostimulatory capacity of DCs used for immunotherapy of MTC.

MENULLARY THYROID carcinoma (MTC) is a calcitonin-producing tumor of the parafollicular C cells that accounts for approximately 5–10% of all thyroid malignancies (1–3). MTC occurs sporadically, in a familial form, or associated with other endocrinopathies such as multiple endocrine neoplasia type 2A or 2B. It has a slow but progressive clinical course with an early involvement of lymph nodes in the neck and mediastinum. In patients with localized disease, the treatment of choice is thyroidectomy with central/lateral neck dissection, whereas effective palliative treatments are lacking once distant metastases have occurred.

We have previously shown that tumor lysate-pulsed dendritic cells (DCs) can stimulate autologous antitumor T cell responses against MTC tumor cells in vitro (4). DCs are regarded as the most potent antigen-presenting cells for naive T cell activation (5, 6). They can be easily generated in vitro from peripheral blood mononuclear cells (PBMCs) using granulocyte-macrophage colony-stimulating factor and IL-4 (7). These cells have the characteristic features of immature DCs, which can be loaded with tumor lysate and further induced to mature by inflammatory stimuli such as TNF-α, IL-1β, or CD40 ligand. Because of their unique capacity to stimulate resting T cells, DCs are a promising option for immunotherapy of patients with various malignancies, including surgically incurable MTC.

In two clinical trials of immunotherapy with autologous tumor lysate-pulsed DCs at our department, we observed objective tumor marker responses and/or disease stabilization in several patients with metastatic MTC (8, 9). Encouraging results were also obtained by Schott et al. (10), who treated seven patients with metastatic MTC and observed clinical and/or tumor marker responses in three patients, including one patient with a complete regression of detectable liver metastases and a significant reduction of pulmonary lesions. However, despite these promising observations, the clinical efficacy of DC immunotherapy in the majority of patients has been limited, suggesting that strategies to further increase the immunostimulatory capacity of DCs are needed to improve overall clinical responses to DC-based immunotherapy in patients with MTC.

Heat-shock proteins (HSPs) have recently been implicated to be potent inducers of innate and antigen-specific immunity (11, 12). HSPs are present in all living cells and function as molecular chaperones in numerous cellular processes un-
under physiological and stress conditions. Several HSPs, including HSP-70 and HSP-90, have been characterized as immunomodulators, owing to their capacity to help the immune system recognize specific antigens, e.g. through cross-presentation of tumor antigens to major histocompatibility complex (MHC) class I molecules or by activating DCs through binding of toll-like receptors (TLRs) on their cell surface (11–20).

Recent investigations using bone-marrow-derived DCs in mice have shown that heat shock increases the expression of HSP-70 and/or HSP-90 by DCs and enhances the capacity of DCs to stimulate antigen-specific T cells (21–23). The mechanisms underlying the enhanced immunostimulatory capacity of the DCs in mice are 1) heat-shock-induced DC maturation with increased expression of costimulatory molecules and 2) enhanced cross-presentation of tumor-derived antigens to MHC class I molecules resulting in an increased cytotoxic antitumor T cell response. However, comparatively little is known about the effects of heat shock on the phenotype and function of monocyte-derived DCs in humans (24). In the present in vitro study, we have investigated whether heat shock enhances the immunostimulatory capacity of monocyte-derived DCs obtained from six patients with metastatic MTC. The aim of our investigations was to obtain initial preclinical evidence that heat shock is suited to enhance the immunostimulatory capacity of tumor lysate-pulsed DCs used for DC-based immunotherapy in patients with MTC.

Patients and Methods

Patients and tumor cell lines

After written informed consent, peripheral blood samples were obtained from six patients with MTC to generate DCs. Genetic tests for known mutations associated with MTC were negative, suggesting that all were sporadic cases: patient 1, 50-yr-old male; patient 2, 47-yr-old male; patient 3, 71-yr-old male; patient 4, 66-yr-old male; patient 5, 31-yr-old female; patient 6, 44-yr-old male. Three allogeneic MTC tumor cell lines were obtained from American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 medium (GIBCO-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (HyClone; Perbio, Logan, UT) and 50 \( \mu \text{g} / \text{ml} \) gentamycin (GIBCO-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (HyClone; Perbio, Logan, UT) and 50 \( \mu \text{g} / \text{ml} \) gentamycin (GIBCO-BRL) in a 5% CO\(_2\) humidified atmosphere at 37°C.

Preparation of DCs from CD14\(^+\) PBMCs

For preparation of DCs, PBMCs were isolated from peripheral blood of patients with MTC using Ficoll-Hypaque (Amersham Pharmacia Biotech AB, Uppsala, Sweden) density-gradient centrifugation. CD14\(^+\) cells were purified using a magnetic bead-conjugated mouse antihuman CD14 monoclonal antibody (CD14-MicroBeads; Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of CD14\(^+\) cells was consistently more than 95%. The cells were seeded at 1 \( \times 10^6 / \text{ml} \) in RPMI 1640 medium supplemented with 500 \( \mu \text{U} / \text{ml} \) granulocyte-macrophage colony-stimulating factor (Leukin Sargramostim; Berlex Laboratories, Richmond, CA), 500 \( \mu \text{U} / \text{ml} \) IL-4 (Strathmann PBH, Hamburg, Germany), 10% heat-inactivated FBS, and 50 \( \mu \text{g} / \text{ml} \) gentamycin in a 5% CO\(_2\) humidified atmosphere at 37°C. On day 2, half the volume of growth medium containing freshly added cytokines was added. On day 5, DCs were pulsed with 100 \( \mu \text{g} / \text{ml} \) tumor cell lysate for 12 h at 37°C (immature tumor lysate-pulsed DCs). Therefore, pulsed DCs were subjected to heat shock for 12 h at 40°C or left untreated. Finally, DCs were matured with 1 \( \mu \text{g} / \text{ml} \) TNF-\( \alpha \) (kindly made available by Dr. H. R. Alexander, Jr., from the National Cancer Institute, Bethesda, MD) plus 1000 \( \mu \text{U} / \text{ml} \) IFN-\( \gamma \) (Imukin; Boehringer Ingelheim, Vienna, Austria) for 12 h, followed by 50 ng/ml lipopolysaccharide (LPS) for 4 h (mature tumor lysate-pulsed DCs).

Preparation of tumor cell lysate

Tumor cells (1 \( \times 10^7 \) to 2 \( \times 10^7 \)) were washed in PBS (GIBCO-BRL) and subjected to five freeze (liquid nitrogen) and thaw (37°C water bath) cycles to obtain a crude lysate. After removal of large particles by centrifugation (2000 \( \times g \) for 10 min at 4°C), the protein content was determined in the supernatant (Coomassie Plus Protein Assay; Pierce, Rockford, IL) and aliquots stored at -80°C until use.

Isolation of autologous T cells

Autologous T cells were isolated by CD3-positive selection from PBMCs using a magnetic bead-conjugated mouse antihuman CD3 antibody (CD3 MicroBeads; Miltenyi Biotec). The purity of isolated T cells was consistently more than 95%.

In vitro cytotoxicity assays

For stimulation of tumor-specific cytotoxic T cells, freshly isolated autologous T cells were cocultured with mature tumor lysate-pulsed DCs at a T:DC ratio of 5:1 in RPMI 1640 plus 10% FBS without addition of cytokines. On day 5, T cells were harvested and T cell-mediated cytotoxicity against MTC tumor cells was measured using a standard in vitro 4-h europium release assay release. Target cells (5 \( \times 10^5 \)) were labeled with europium for 15 min at room temperature. Subsequently, 5 \( \times 10^5 \) target cells and serial dilutions of effector cells at effectortarget ratios ranging from 50:1 to 3:1 were incubated for 4 h in 200 \( \mu \text{l} \) RPMI 1640 medium (without phenol red) and 10% FBS in round-bottom 96-well plates. Twenty-five microliters of the supernatant were harvested, and europium release was measured by time-resolved fluorometry (Wallac, Turku, Finland). Specific cytotoxic activity was calculated by the following formula: percent specific release = (experimental release – spontaneous release)/(total release – spontaneous release) \( \times 100 \). Spontaneous release of the target cells was generally less than 25% of total release as determined by detergent (2% Triton X-100).

Flow cytometry

The phenotype of DCs was determined using fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated mouse antibodies against human CD1a, CD11c, CD40, CD80, CD86, human leukocyte antigen (HLA)-ABC, and HLA-DR. Appropriate mouse IgG isotype controls were used to determine the levels of background staining. Analysis for surface HSP-70 expression was performed using an anti-HSP-70 mouse antihuman monoclonal IgG2a antibody (Santa Cruz Biotechnology, Santa Cruz, CA) followed by a FITC-conjugated secondary antibody. Analysis for expression of TLR-2 and TLR-4 was performed using anti-TLR-2 and -TLR-4 mouse antihuman antibodies (eBioscience, San Diego, CA) and subjected to five freeze (liquid nitrogen) and thaw (37°C water bath) cycles to obtain a crude lysate. After removal of large particles by centrifugation (2000 \( \times g \) for 10 min at 4°C), the protein content was determined in the supernatant (Coomassie Plus Protein Assay; Pierce, Rockford, IL) and aliquots stored at -80°C until use.

Western blot

Cell extracts were prepared in Strong Lysis Solution (BIOZOL Diagnostica, Eching, Germany). For detection of HSP-70, 15 \( \mu \text{g} \) cell extract was separated by SDS-PAGE and electrophoretically transferred onto a nitrocellulose membrane (Invitrogen Life Technologies, Carlsbad, CA). After blocking with BSA (Sigma-Aldrich, St. Louis, MO), the membrane was incubated with an HSP-70 mouse antihuman monoclonal IgG2a antibody (Santa Cruz Biotechnology, Santa Cruz, CA) followed by a FITC-conjugated secondary antibody. For detection of TLR-2 and TLR-4 was performed using an anti-TLR-2 and -TLR-4 mouse antihuman antibodies (eBioscience, San Diego, CA) followed by a PE-conjugated secondary antibody. Approximately 10,000 cells per sample were analyzed using an Epics XL flow cytometer (Beckman Coulter, Fullerton, CA).

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Confocal microscopy

DCs were cultured and matured using chamber slides. Mature DCs were fixed with acetone. After blocking with BSA, the slides were incubated with a mouse antihuman HSP-70 antibody followed by a secondary goat antimouse Alexa Fluor 568 antibody (Molecular Probes, Eugene, OR). After several washing steps, the slides were incubated with a mouse antihuman MHC class I antibody followed by a secondary goat antimouse Alexa Fluor 568 antibody (Molecular Probes). Nuclear staining was performed using TO-PRO 3 (Molecular Probes). To elicit immunofluorescent staining, argon 488-nm and helium/neon 633-nm and 543-nm lasers were used. Immunofluorescent staining was evaluated using a Zeiss LSM 510 laser scanning confocal microscope.

HLA typing

HLA typing of patients and tumor cell lines was performed using serological typing (patients 1, 3, 5, and 6) or using the DNA-based PCR-sequence-specific oligonucleotide probes (SSOP) technique (patients 2 and 4; tumor cell lines GSJO, KJOS, SHER, and PANC-1) as previously described (26–28).

Statistics

To determine differences between the cytotoxic activity of T cells cocultured with heat-shocked and control DCs, two-sided Student’s t test was used. A P value less than 0.05 was considered statistically significant.

Results

Heat shock induces enhanced HSP-70 expression by immature DCs, whereas HSP-90 expression remains largely unaffected by heat shock

To assess the effect of heat shock on HSP-70 and HSP-90 expression by DCs, immature DCs were exposed to temperatures of 37, 38, 39, 40, or 41 C for 3, 6, 12, or 24 h, followed by a recovery period at 37 C overnight. Thereafter, the expression of HSP-70 and HSP-90 was assessed using Western blot. As shown in Fig. 1A, immature DCs kept at 37 C constitutively expressed both HSP-70 and HSP-90. After heat-shock treatment, a marked enhancement of HSP-70 expression by the DCs was observed, whereas HSP-90 expression remained largely unaffected by heat shock. The effect of heat shock on HSP-70 expression was both temperature and time dependent. For subsequent experiments, we used a 12-h heat shock at 40 C because longer (24 h) exposure to 40 C did not further increase HSP-70 expression, whereas exposure to 41 C led to decreased viability of the DCs (data not shown).

Heat-shock-mediated up-regulation of HSP-70 is not abrogated by DC maturation

Next, we tested whether up-regulation of HSP-70 is sustained after maturation of DCs. Immature heat-shocked DCs were matured with TNF-α, IFN-γ, and LPS, followed by Western blot analysis for HSP-70. As shown in Fig. 1B, both immature and mature heat-shocked DCs displayed increased amounts of HSP-70, indicating that the enhanced expression of HSP-70 by DCs is not abrogated by subsequent DC maturation. Enhanced HSP-70 expression was seen both in mature DCs derived from healthy donors as well as patients with MTC.

Heat shock leads to HSP-70 up-regulation on the surface of DCs

Using flow cytometry, we subsequently assessed whether HSP-70 was also expressed on the cell surface of the DCs. As shown in Fig. 2A, immature and mature DCs kept at 37 C expressed only minimal levels of surface HSP-70 (suggesting that the constitutive expression of HSP-70 by the DCs shown in Fig. 1A occurs predominantly intracellularly), whereas heat-shocked DCs displayed a markedly enhanced expression of surface HSP-70. Notably, surface HSP-70 expression was highest on mature heat-shocked DCs.

Heat shock of immature DCs does not induce DC maturation

Next, we assessed whether heat shock itself influences the maturation status of the DCs. As shown in Fig. 2B, immature control DCs did not express the DC maturation marker CD83, as determined by flow cytometry. CD83 expression remained unchanged after heat shock of immature DCs, indicating that heat shock did not induce DC maturation. DCs that had been matured with TNF-α, IFN-γ, and LPS displayed the characteristic up-regulation of CD83, with control DCs displaying a somewhat higher level of CD83 than heat-shocked DCs.

Mature heat-shocked DCs coexpress HSP-70 and MHC class I molecules

Because HSP-70 has been implicated to play a pivotal role for cross-presentation of tumor antigens to MHC class I molecules, we evaluated HSP-70 and MHC class I expression by...
DCs using confocal microscopy. Importantly, mature heat-shocked DCs displayed an even distribution and coexpression of HSP-70 and MHC class I molecules, whereas no coexpression was found in mature control DCs (Fig. 3).

**Heat-shocked DCs display increased surface expression of TLR-2 and TLR-4**

Next, we assessed the effect of heat shock on the expression of TLR-2 and TLR-4 by immature DCs using flow cytometry. As shown in Fig. 4, immature DCs kept at 37°C displayed only low expression of both TLR-2 and TLR-4 on their cell surface. After heat shock of the DCs, the percentage of cells staining positive for TLR-2 increased 2.7-fold from 8.8 to 23.5%, and the percentage of cells staining positive for TLR-4 increased 2.5-fold from 5.4 to 13.4%.

**Heat-shocked DCs are more potent stimulators of cytotoxic T cell responses than control DCs**

We finally assessed the immunostimulatory capacity of the DCs to elicit cytotoxic T cell responses against solid tumor cells (Fig. 5). DCs and T cells were derived from six patients with metastatic MTC. T cells were cocultured with mature DCs (heat-shocked vs. control) that had been loaded with tumor lysate derived from one of three different allogeneic MTC cell lines (GSJO, KJOS, and SHER). Unstimulated T cells were used as a control. Table 1 shows the HLA type for all patients and tumor cell lines. After coculture of DCs with T cells, the cytotoxic activity of the T cells was tested either against the MTC cell line from which the tumor lysate had been derived for DC loading or against an MTC cell line unrelated to the respective tumor cell lysate or PANC-1 pancreatic cancer cells for control. Figure 5 shows that in all patients, cytotoxic T cell responses against the MTC cell line from which the tumor lysate had been derived for DC loading could be induced (left column of graphs). Notably, heat-shocked DCs were more potent stimulators of cytotoxic T cell responses than control DCs: using heat-shocked DCs as stimulators, the mean cytotoxic activity of T cells could be increased significantly from 15.3% (range, 2.02–26.8%) to 20.1% (range, 4.8–35.6%; P = 0.011; effector:target ratio, 25:1) and from 23.6% (range, 3.7–41.8%) to 28.2% (range, 12.1–51.6%; P = 0.040; effector:target ratio, 50:1) compared with T cells stimulated with control DCs. Importantly, in none of the patients was a relevant cytotoxic T cell response against PANC-1 cells observed, using both control and heat-shocked DCs (right column of graphs). Also, cytotoxic T cell responses against unrelated MTC tumor cells (middle column of graphs) were consistently lower than the cytotoxic responses induced by the tumor lysate-pulsed DCs were tumor cell specific.
Discussion

In the present in vitro study, we show that heat shock increases the efficacy of tumor lysate-pulsed DCs from patients with MTC to stimulate cytotoxic T cell responses against MTC tumor cells. We provide evidence that the increased T cell stimulatory capacity of heat-shocked DCs is tumor antigen specific because heat-shocked DCs pulsed with tumor cell lysate derived from MTC tumor cells do not stimulate cytotoxic T cell responses against tumor cells from a different tumor entity (PANC-1). We demonstrate that the enhanced T cell stimulatory capacity of heat-shocked DCs is accompanied by up-regulation of HSP-70 and coexpression of HSP-70 with MHC class I molecules, whereas HSP-90 expression remains largely unaffected by heat shock. We also show heat shock of immature DCs results in enhanced expression of immunoadjuvant surface receptors TLR-2 and TLR-4, which activate DCs through binding of exogenous and endogenous ligands, including HSPs.

Using an autologous in vitro MTC model, we have previously shown that tumor lysate-pulsed DCs can stimulate MHC class I-restricted cytotoxic T cell responses against MTC cells (4). In the present study, we have addressed the
question whether the T cell stimulatory capacity of the DCs can be further enhanced by heat shocking the DCs before T cell stimulation. The rationale for this approach is based on other in vitro studies using bone-marrow-derived DCs in mice that showed that heat shock increases the expression of HSP-70 and/or HSP-90 by DCs and enhances the capacity of DCs to stimulate antigen-specific T cells (21–23). Our study confirms the above-mentioned data using monocyte-derived DCs in a human in vitro MTC model by showing 1) a heat-shock-induced up-regulation of HSP-70 by the DCs, 2) a heat-shock-induced enhanced capacity of the DCs to stimulate cytotoxic T cell responses against MTC cells, and 3) antigen specificity of the triggered cytotoxic effect.

Previous investigations have largely focused on heat shocking tumor cells and loading DCs with tumor antigens derived from the heat-shocked tumor cells to enhance antitumor T cell responses (29–33). It has been shown that HSPs that are up-regulated by the tumor cells after heat shock induce DC maturation and enhance antigen cross-presentation to MHC class I molecules by the DCs. This results in an improved T cell stimulatory capacity of the DCs and an enhanced cytotoxic antitumor T cell response. However, such an approach requires the availability of a tumor cell line that can be heat shocked before obtaining a crude tumor cell lysate for loading the DCs. Although it has been shown that MTC tumor cell lines can be established in the laboratory from surgical specimens of MTC, the procedure is time consuming and not successful in every case (25, 34–36). Obviously, this limits the clinical applicability of heat shocking tumor cells within a clinical protocol of DC immunotherapy. In contrast, heat shocking tumor lysate-pulsed DCs with the intention to increase their T cell stimulatory capacity can be easily performed without significant additional laboratory expenditure.

We have found that heat shock of human monocyte-derived DCs results in up-regulation of HSP-70, which is known to play a pivotal role for cross-presentation of tumor antigens via MHC class I molecules to CD8+ cytotoxic T cells (12, 37). Flow cytometric analysis revealed up-regulation of HSP-70 on the surface of DCs, and additional confocal microscopy showed coexpression of HSP-70 and MHC class I molecules. It seems likely that an interaction of HSP-70 with MHC class I molecules led to a better ability of heat-shocked DCs to present tumor-derived antigens to cytotoxic T cells, which resulted in an enhanced cytotoxic T cell response against MTC tumor cells. Another mechanism that may have contributed to the enhanced T cell stimulatory capacity of heat-shocked DCs is the formation of DC aggresome-like induced structures (DALISs). DALISs have previously been described in bone-marrow-derived DCs in mice and are thought to play an important role for loading antigens onto MHC class I molecules. DALISs are typically found in mature DCs and have been shown to be induced by various stimuli, including heat shock (22, 38).

We were also able to show that heat shock induces enhanced expression of TLR-2 and TLR-4 by immature DCs. TLRs are type I integral membrane receptors on antigen-presenting cells that recognize microorganism-associated molecular patterns such as LPS, bacterial DNA, and viral RNA and display potent immunoadjuvant capacity (39, 40). HSPs have recently been shown to be potent endogenous ligands of TLRs, capable of activating immature DCs (17–20). We show that heat shock of immature DCs results in enhanced expression of TLR-2 and TLR-4 on the surface of the DCs. Conceivably, interaction of HSP-70 with TLRs could serve as an additional activation signal of immature DCs and further explain the superior immunostimulatory capacity of heat-shocked DCs as compared with DCs kept at 37°C.

In contrast to previous studies using bone-marrow-derived DCs in mice, heat shock did not induce DC maturation in our in vitro system (21). Similarly, Ostberg and Repasky (24) reported that heat shock of human monocyte-derived DCs does not result in maturation of DCs. Conceivably, this may be explained by species- or cell-type-specific differences between monocyte-derived human DCs and bone-marrow-derived mouse DCs. However, heat shock did not abrogate the ability of the monocyte-derived DCs to undergo maturation upon additional stimulation with TNF-α, IFN-γ, and LPS, as evidenced by similar expression levels of the DC maturation marker CD83 on heat-shocked and control DCs. Also, maturation of the DCs did not abrogate the enhanced expression of HSP-70 on the surface of DCs. These findings are particularly important in our opinion, given the fact that mature DCs should be given preference over immature DCs for DC-based cancer immunotherapy.

In conclusion, our data show that heat shock is a promising strategy to increase the immunostimulatory capacity of tumor lysate-pulsed DCs used for immunotherapy of MTC. Considering the little additional laboratory expenditure required for heat shocking DCs, an in vitro heat-shock step could be easily introduced into DC immunotherapy protocols. Given results such as those of a recent phase III trial in patients with stage IV melanoma where autologous peptide-loaded DCs were not significantly better than standard dacarbazine chemotherapy, strategies to increase the clinical efficacy of DCs are needed to ensure the future role of DC immunotherapy in cancer patients (27). Even though the heat-shock-mediated increase of the immunostimulatory capacity of DCs may appear to be small, it may be a crucial step to overcome immune tolerance in vivo in MTC patients. Therefore, future studies on the effect of hyperthermia on DC function are clearly warranted, in our opinion.

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Table 1. HLA type of patients and tumor cell lines

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Bachleitner-Hofmann et al. • Heat Shock Enhances DC Function in MTC

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