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Generation of myeloma-specific T cells using dendritic cells loaded with MUC1- and hTERT- drived nonapeptides or myeloma cell apoptotic bodies

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Dendritic cells are able to induce anti-tumor immune responses by presenting tumor-specific antigens to T-lymphocytes. Various tumor-associated antigens have been studied in multiple myeloma in an effort to find a strong antigen capable of generating clinically meaningful responses in vaccinated patients. The aim of our study was to generate myeloma-specific cytotoxic T lymphocytes *in vitro* using dendritic cells loaded with peptide antigens or apoptotic bodies.

Peripheral blood mononuclear cells from HLA-A2+ healthy donors were used for isolation and culture of dendritic cells (DCs) and T lymphocytes. DCs were loaded with hTERT- and MUC1-derived nonapeptides or apoptotic bodies from myeloma cells. Repeated stimulation of T lymphocytes led to their activation characterized by interferon-gamma production. Activated T lymphocytes were separated immunomagnetically and expanded i*n vitro*. Specific cytotoxicity of the expanded T lymphocytes was tested against a myeloma cell line. There was evidence of cytotoxicity for all three types of antigens used for T lymphocyte priming and expansion. No statistically significant differences were observed in T lymphocyte cytotoxicity for any of the antigens.

We present a method for the priming and expansion of myeloma-specific T lymphocytes using dendritic cells loaded with different types of tumor antigens. Cytotoxic T lymphocytes and/or activated dendritic cells generated by the described methods can be applied for cellular immunotherapy against multiple myeloma and other malignancies.

Keywords: multiple myeloma, antitumor immunotherapy, dendritic cells, hTERT, MUC1, apoptotic bodies

Multiple myeloma (MM) is an incurable disease which nevertheless can be controlled over long periods of time using modern treatment strategies [1]. Standart therapy for MM includes high-dose chemotherapy with autologous stem cell transplantation [2]. More recently, novel agents have become a part of the therapeutic armamentarium, including immunomodulatory drugs such as thalidomide and its more potent analogue lenalomide and bortezomib, a proteasome inhibitor, [3–6]. However new anti-myeloma treatment strategies are needed, because even if complete remission (CR) is achieved, relapse of MM is inevitable [7, 8].

Antineoplastic immunotherapy has been somewhat successful in a few types of malignancies, especially melanoma and renal cell carcinoma (RCC) [9, 10]. Over the past 15 years, there have been experimental studies suggesting the feasibility of this approach in MM. Potentially clinically meaningful immune responses have been induced in this setting albeit the disease is less immunogenic than melanoma or RCC [11, 12]. Anti-tumor vaccination is usually well tolerated by the patients and the side effects are minimum [9, 13–21].

Immune response against tumor-specific antigens can be activated using antigen-loaded dendritic cells (DCs). DCs prime naive T-lymphocytes, generating tumor-specific T lymphocytes capable of killing malignant cells [22].

Anti-myeloma immunotherapy usually utilizes DCs loaded with myeloma-specific antigens such as idiotype (Id) protein or myeloma cell lysate [23, 24]. Currently, several other sources of tumor-associated antigens are studied, including myeloma

cell-derived apoptotic bodies, and tumor-specific proteins such as hTERT and MUC1 [14, 16, 17, 18, 25].

hTERT is the catalytic subunit of telomerase, an enzyme synthesizing terminal parts of eukaryotic chromosomes called telomeres. Telomeres serve to protect chromosomes and have a role in cell proliferation [26, 27]. There is a tight correlation between telomerase activity and malignant potential of tumor cells. Telomerase activity in a cell is associated with the expression of hTERT-related peptides on its surface [28] and is present in more than 85% of human tumors [29].

It has been shown previously that hTERT can be a target for cytotoxic T lymphocytes in several malignancies including MM *in vitro* [30, 31] and *in vivo* [26, 32]. hTERT-specific T lymphocytes have also been identified in healthy individuals [13, 14, 16]. No autoimmune side effects have been reported after anti-hTERT vaccination [13, 14, 16]. Immunological responses and some clinical responses were seen in recent clinical studies of antitumor vaccines that use hTERT as the tumor antigen in different types of malignancies [14, 16]. In seven vaccinated patients with prostate cancer and breast cancer, one mixed response and four stable disease responses were reported [14]. Of 27 vaccinated patients with RCC, 13 responded with stable disease [16]. For our experiments, we have used the HLA-A2 specific nonapeptide hTERT I_{540} (amino acid sequence ILAKFLHWL).

MUC1 is type I transmembrane glycoprotein expressed on epithelial and some non-epithelial tumor cells. Its extracellular domain is composed of a variable number (20–60) of tandem repeats of 20 amino acids (tandem repeat domain; TRD) [15, 33]. Antigenic epitopes are contained within the TRD [34]. MUC1 is amply expressed in hematological malignancies, especially in MM (92%) [25, 35] as well as in RCC, breast and prostate carcinoma [17, 18]. It is also expressed by HLA-A2+ myeloma cell line ARH 77 [36, 37]. Immunization with MUC1 protein results in activation of cytotoxic T lymphocytes both *in vitro* and *in vivo* [15, 35, 38, 39].

Clinical studies of DCs loaded with MUC1-derived nonapeptides have been carried out in various malignancies including RCC, breast and prostate cancer [17, 18]. The vaccine was well tolerated and immunological responses were detected 55% (11/20) of RCC patients. Thirty percent (6/20) of patients had regression of metastases, including 5% (1/20) patients achieving complete response (CR) and 10% (2/20) patients with partial response (PR) [17, 18].

Although MUC1 is also expressed by some normal cells (e.g. gastrointestinal epithelial cells, hematopoietic cells of the bone marrow), no autoimmune reactions were observed during MUC1-directed vaccination studies [19]. In our study we have used the HLA-A2-specific MUC1-derived nonapeptide with amino acid sequence TSAPDTRPA [40, 41].

In contrast to antigenic peptides, apoptotic bodies prepared by irradiation of myeloma cells are a source of complex antigenic structures. After loading onto DCs, apoptotic bodies have been shown to generate myeloma-specific cytotoxic T lymphocytes *in vitro* [42]. DCs loaded with apoptotic bodies have been administered to patients with MM and B-cell chronic lymphocytic leukemia without any significant adverse effects [19, 20]. Tumor responses in MM patients included CR in 35%, PR in 29%, and SD in 18% of patients; 12% of vaccinated patients had disease progression [21].

No studies comparing the efficacy of tumor-associated peptides versus apoptotic bodies loaded onto DCs have been published so far. The aim of our study was to compare their respective potential to induce specific cytotoxicity directed against myeloma cells. DCs loaded with HLA-A2 specific nonapeptides hTERT I_{540} and MUC1 or apoptotic bodies from ARH 77 myeloma cell line were used to prime and restimulate T lymphocytes. We show that these antigen–specific T lymphocytes produce interferon gamma (IFN-**γ**) and exhibit specific cytotoxicity against myeloma cells.

Materials and methods

Cell cultures. Peripheral blood mononuclear cells. Peripheral blood mononuclear cells (PBMCs) were isolated from anticoagulated peripheral blood of HLA-A2+ healthy donors from the transfusion facility of the Brno University Hospital. Prior to blood collection, all donors had signed informed consent approved by the local ethical committee. Buffy coats were processed by gradient centrifugation (using Histopaque 1077, Sigma-Aldrich, Prague, Czech Republic) and cultured in a medium containing X-VIVO 10 (BioWhittaker, Walkersville, MD, USA) with heat-inactivated 10% human AB serum (Sigma-Aldrich, Prague, Czech Republic), 80 U/ml DNAse (Boehringer, Mannheim, Germany) and 1 mM L-glutamine (Sigma-Aldrich, Prague, Czech Republic) in 6-well plates at 37°C, 5% CO_2, and 4.5% O_2. Cells were seeded at a concentration of $3.3x10^6$ PBMCs per ml of medium.

T lymphocytes. After 2-hour culture of PBMCs, non-adherent cells were collected. This population is known to contain predominantly T lymphocytes. Non-adherent cells were subsequently cultured in complete medium (CM) containing X-VIVO 15, 50 mg/l gentamycin, 2 mM L-glutamine, 25 mM HEPES buffer (BioWhittaker, Walkersville, MD, USA), 10% human AB-serum (Sigma-Aldrich, Prague, Czech Republic), and 10 IU/ml interleukin-2 (IL-2) [Proleukin, Chiron, Amsterdam, Holland] for 7 days at 37°C and 5% CO_2. Cell concentration was $1x10^6$ T lymphocytes per ml of CM.

Dendritic cells Adherent cells containing DC precursors were cultured in DC culture medium consisting of X-VIVO 10 (BioWhittaker, Walkersville, MD, USA) with heat-inactivated 2% human serum (Sigma-Aldrich, Prague, Czech Republic), 20 ng/ml interleukin 4 (IL-4) (CellGenix, Freiburg, Germany), 100 ng/ml granulocyte and macrophage colony-stimulating factor (GM-CSF) [CellGenix, Freiburg, Germany] for 6 days at 37°C, 5% CO_2, and 4.5% O_2. The medium including IL-4 and GM-CSF was changed on days 3 and 6 of culture. On day 6, maturation cytokine mix was added, containing 10 ng/ml tumor necrosis factor-alpha (TNF-α) [Bender Medsystems Diagnostics, Vienna, Austria], 10 ng/ml IL-1β (CellGenix,

Freiburg, Germany), 250 ng/ml prostaglandin E_2 (PGE_2; Prostin, Dinoprostone, Pfizer Inc., New York, USA), 1 μg/ml Toll-like receptor (TLR) ligand R848 (InVivogen, California, USA), and 5000 IU/ml IFN-γ (Gentaur, Brussels, Belgium) [43, 44]. Some of the immature DCs were loaded with apoptotic bodies of ARH 77 myeloma cell line (apo ARH 77). The remaining DCs were allowed to differentiate into mature DCs for 24 h at 37°C, 5% CO_2, and 4.5% O_2 and then loaded with antigenic peptides (see section 2.3.).

Antigen preparation. Apoptotic bodies were produced from ARH77 myeloma cell line and used DC loading (ARH 77 cell line was kindly donated by professor B Barlogie University of Arkansas, USA). Apoptosis was induced by irradiating ARH 77 cells to a dose of 60 Gy (using Gammacell 1000 Elite blood irradiator at the transfusion facility, Brno University Hospital).

Tumor-specific peptides were used also as antigens. The I_{540} nonapeptide is derived from the catalytic subunit of hTERT telomerase and its amino acid sequence is IL-AKFLHWL [29]. Another used nonapeptide was derived from MUC1 protein and its aminoacid sequence was TSAPDTRPA [17] [Proimmune, Oxford, Great Britain]. Non-stimulating ^{476}ILKEPVHGV^{484} nonapeptide derived from HIV-1 reverse transcriptase was used as a negative control [Proimmune, Oxford, Great Britain]. All three nonapeptides are specific for HLA-A2 [17, 29]. Peptides were dissolved in sterile water to a concentration of 1 mg/ml prior to adding to cell cultures.

Loading of DCs with tumor antigens On day 6 of culture, maturation-inducing cytokines (TNF-α, IL-1β, IFN-γ, TLR-R848 a PGE_2) were added to immature DCs and a portion of DCs were loaded with apo ARH 77 in 1:1 ratio. These cells were designated L (loaded) cells for the purpose of immunophenotyping. All DCs were then cultured for 24 h at 37%, 5% CO_2, and 4.5% O_2 in order to produce mature DCs.

On day 7 of culture, hTERT, MUC1, and HIV-1 nonapeptides in an amount of 10 **μ**g peptide/100,000 DCs were added to mature unloaded DCs (designated as NL: non-loaded) (17). DCs were collected and counted after further 2h of culture. Mature DCs loaded with different antigens (hTERT, MUC1, HIV-1, or apo ARH 77) were used immediately for T lymphocyte stimulation, with an aliquot frozen at -80°C for later restimulation of T cell cultures. Mann – Whitney Test was used for statistical analysis.

Stimulation and restimulation of T lymphocytes. On day 7, mature DCs loaded with different antigens (hTERT, MUC1, HIV-1, or apo ARH 77) were mixed with T lymphocytes ($1x10^6$ T lymphocytes/ml CM) in a ratio of 20:1 (T lymphocytes:DCs) and cultured for 7 days at 37°C and 5% CO_2.

Seven days after the first stimulation, restimulation was carried out using thawed DCs that were added to T lymphocyte cultures in a ratio of 2:1 (T lymphocytes:DCs) as published previously [45]. After further 24h of culture, T lymphocytes were harvested and activated IFN-**γ**-producing T lymphocytes were isolated by immunomagnetic separation.

Detection and immunomagnetic separation of activated T lymphocytes. IFN-**γ** -producing subpopulation of T lymphocytes were identified using the Secretion Assay Cell Enrichment and Detection Kit (MACS Reagens, Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions [45, 46] and analyzed using a flowcytometer. T lymphocytes primed with DCs loaded with HIV-1 antigen were used as a negative control. After labeling with paramagnetic microbeads, the cells were placed into the magnetic field of the Vario MACS apparatus (MACS Reagents, Miltenyi Biotec, Bergisch Gladbach, Germany), and the positive fraction was separated according to the manufacturer's instructions [45, 46].

The purity of the positive fraction was increased by double separation. The efficacy of separation was verified using flow cytometry and T lymphocytes from the positive fraction were subsequently expanded *in vitro.*

Flow cytometry. The phenotype of PBMCs on day 0 and of mature NL DCs and L DCs on day 7 was analyzed using the CytomicsTM FC 500 flow cytometer (Beckman Coulter, Hialeah, USA). We have evaluated the expression of the following surface antigens: CD3 and CD14 to identify T lymphocyte and monocyte populations; HLA-DR, a marker of professional antigen-processing cells; CD83, a marker of DC maturation status contributing to interaction with T lymphocytes [47]; CD80 and CD86, costimulation molecules. We also analyzed HLA-DR/CD80 and HLA-DR/CD86 marker combinations [48]. Cell viability was assessed by propidium iodide (PI) staining. Cells (0.5×10^6) were incubated with appropriate monoclonal antibodies labeled with fluoroisothiocyanate (FITC), phycoerythrin (PE), or phycoerythrin-cyanin 5 (PC-5) for 15 min. PBS stabilized with sodium azide was then added and the cells were fixed with 1% paraformaldehyde. We have used the following labeled antibodies: CD83-PC5, HLA-DR-FITC, CD80-PE, CD86-PE, and CD14-PE, and combinations HLA-DR-FITC/CD80-PE and HLA-DR-FITC/CD86-PE (Immunotech, Marseille, France).

T lymphocytes ($1x10^6$) after restimulation with loaded DCs were incubated for 15 min with the following monoclonal antibodies: CD4-FITC, CD8-FITC, CD3-PE-Cy (Immunotech, Marseille, France), and IFN-**γ**-PE (Miltenyi Biotec, Bergisch Gladbach, Germany). T lymphocytes were analyzed in the cell suspension prior to immunomagnetic separation and in the positive and negative fractions after the separation. Non-stimulated T lymphocytes and T lymphocytes primed with non-stimulating HIV-1 peptide-loaded DCs were used as negative controls.

Expansion of IFN-γ$^+$ antigen-specific T lymphocytes. IFN-**γ**$^+$ hTERT/MUC1/apo ARH 77 –specific T lymphocytes after immunomagnetic separation were expanded *in vitro* in CM enriched with IL-2 in a concentration of 100 IU/ml of CM (Proleukin, Chiron, Amsterdam, The Netherlands). Microbeads conjugated with anti-CD3 and anti-CD28 antibodies were used for the expansion (Invitrogen, California, USA) in a quantity of 75 μl per 1×10^6 of T lymphocytes according

to the manufacturer's instructions. The initial concentration of T lymphocytes was 1 x 10^5 T lymphocytes/100 μl of CM per well in a 96-well plate. Cells were counted twice weekly (starting on day 4 or 5 of culture) and maintained at the concentration of 1-5 x 10^6 T lymphocytes/ml for 2 to 3 weeks. IL-2 in a quantity of 100 IU/ml was added 2 to 3 times weekly. After 14 days of culture, if further expansion was necessary, another aliquot of microbeads was added.

Cytotoxicity test. The cytotoxicity potential of expanded antigen-specific T lymphocytes was assessed using the LIVE/DEAD® flowcytometric non-radioactive cytotoxicity test (Cell-Mediated Cytotoxicity Kit, Invitrogen Corporation, Carlsbad, USA). The flowcytometric cytotoxicity assay [49] used in our study has been shown to be quite specific and reproducible. The 3,3'-dioctadecyloxacarbocyanine perchlorate [$DiOC_{18}(3)$] staining allows labeling of any cell population and does not interfere with other monoclonal antibodies used for flowcytometric characterization of effector or target cell populations. [$DiOC_{18}(3)$] labeling is stable enough to be used in assays lasting for up to 8 days, and importantly, [$DiOC_{18}(3)$] in applied concentrations is non-toxic and does not interfere with lytic properties or viability of labeled cells [49, 50, 51].

ARH 77 myeloma cells expressing hTERT and MUC1 antigens were labeled with fluorescent dye [$DiOC_{18}(3)$] according to the manufacturer's instruction (49) and used as target cells. Expanded T lymphocytes specific for the appropriate antigen (hTERT, MUC1, apo ARH 77) were used as effector cells. Effector and target cells were mixed in 20:1 and 40:1 ratios (T lymphocytes:ARH 77 cells) and incubated for 4h at 37°C and 5% CO_2. Target cell lysis was assessed using after PI staining [50].

As a negative control, the same effector cells were incubated with allogeneic PBMCs in effector:target ratios as above [51]. Cell-mediated killing was quantified using the following formula [52]:

$$\text{cytotoxicity (\%)} = \frac{(\text{mixture of effectors and targets-effector control}) \times 100}{(\text{maximum-spontaneous})}$$

Statistical analysis. Data were expressed as median, minimum, and maximum values. Experiment results were analyzed using SPSS 16.0 statistical package. Differences among groups were assessed by the Kruskal-Wallis and unpaired Mann-Whitney tests. $P < 0.05$ was considered statistically significant.

Results

Dendritic cell culture. DCs were successfully produced culture from peripheral blood-derived precursors in all experiments. Immunophenotype of immature and mature DCs is described in Figure 1. After 7 days of culture, $CD14^+$ monocytes differentiated into immature DCs concurrently with gradual decrease of $CD3^+$ T lymphocyte count (data not shown). PBMC/DC viability was 98.2-99.5 % (median 98.7%) on day 0, and 84.8-94.3% (median 87.1%) on day 7. DC maturation was reflected by increasing expression of certain surface antigens, including HLA-DR, CD80, CD86, CD83 and their combinations such as HLA-DR/CD80 and HLA-DR/CD86. Median expressions of these antigens on days 0 and 7 of culture together with maximum and minimum values are shown in Figure 1. The percentage of cell expressing HLA-DR/CD80 ranged from 0.2 to 0.9 % (median 0.6 %) on day 0, while it was 87.0 to 99.5 % (median 92.0 %) on day 7. Similarly, the proportion of cells expressing HLA-DR/CD86 was 8.0 to 18.0 % (median 10.0 %) on day 0, and it increased to 88.0-99.5 % (median 92.3 %) on day 7.

The increase in the expression of surface antigens including HLA-DR, CD80, CD86, HLA-DR/CD80, and HLA-DR/CD86 between days 0 and 7 of culture was higher in mature apo ARH 77 –loaded DCs (L DCs; *loaded*) than in non-loaded mature DCs (NL DCs; *non loaded*). The increase in the expression of these antigens in L DCs ranged from 80.4 to 91.6 % (median 82.3 %) while in NL DCs the increase was by 75.2 to 88.1 % (median 79.9%). This correlates with the status of cells, because L cells were already loaded with antigen (apo ARH 77) before immunophenotyping, while NL cells were loaded with antigenic nonapeptide subsequent to their immunophenotypic analysis. Nevertheless, both L and NL DCs exhibited significant immunophenotypic changes during culture reflecting their differentiation and maturation (Figure 1). No statistically significant differences in the expression of the above surface antigens between mature NL and L DCs were seen (Figure 1).

Flowcytometric analysis was carried out on days 0 and 7 for the following surface markers of DC maturation: CD83 (a marker of DC maturation status), CD80 and CD86 (costimulatory molecules), HLA-DR (antigen-presenting function), and HLA-DR/CD80 and HLA-DR/CD86 combinations.

Identification of IFN-γ^+ antigen- specific T lymphocytes. On day 7 of culture, DCs loaded with apo ARH 77 or hTERT, MUC1, and HIV-1 nonapeptides were used to stimulate autologous T lymphocytes in a 20:1 ratio (T lymphocytes:DCs). Aliquots of loaded DCs were frozen for later restimulation (45). After further 7 days of culture, T lymphocytes were restimulated using thawed antigen-loaded DCs added in a 2:1 ratio (T lymphocytes:DC) and cultured for another 24h. The ratios used for stimulation and restimulation had been optimized earlier (45, 51) and shown to lead to optimal T lymphocyte stimulation. Activation of anti-tumor myeloma-specific T lymphocytes was evaluated based on IFN-γ production for $CD4^+$ and $CD8^+$ T lymphocytes (Figure 2). T lymphocytes restimulated with HIV-1 nonapeptide-loaded DCs were used as a negative control. The percentage of activated IFN-γ^+ T lymphocytes for all three antigens (i.e., apo ARH 77, hTERT a MUC1 nonapeptides) was 0.87-1.74 % (median 1.28 %) for $CD4^+$ and 0.78-1.43% (median 1.14 %) for $CD8^+$ T lymphocytes prior to immunomagnetic selection (Figure 2).

The percentage of T lymphocytes activated using DCs loaded with apo ARH 77 or hTERT- and MUC1-derived nonapeptides was significantly higher for both CD4+ and CD8+ T lymphocytes as compared to T lymphocytes activated with nonstimulating HIV-1 peptide-loaded DCs used as a negative

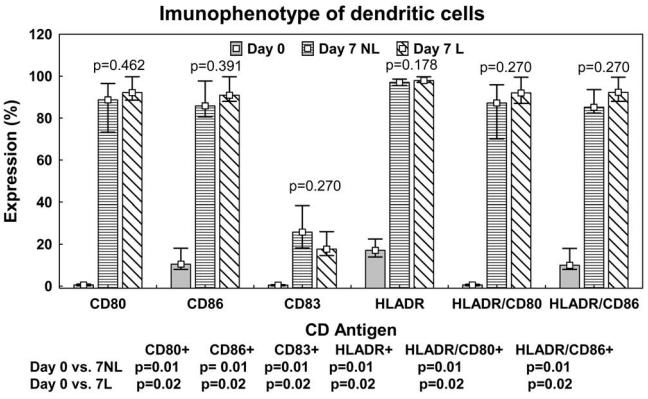

Fig.1: Immunophenotyping of dendritic cells

L cells: mature DCs loaded with apo ARH 77; NL cells: mature non-loaded DCs prior to loading with hTERT, MUC1 or HIV-1 nonapeptides. Five independent experiments were carried out (P1 – P5). The figure shows median, minimum and maximum values (%) and statistical significance of differences calculated using the Mann-Whitney test. Differences inn surface antigen expression between immature (day 0) and mature (day 7) L/NL DCs were statistically significant while differences between mature (day 7) L and NL cells were not statistically significant.

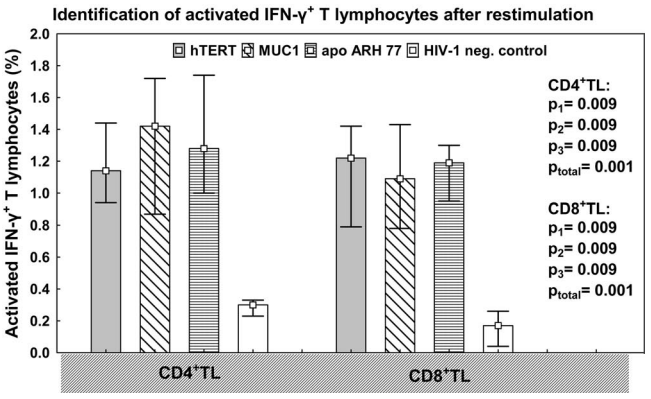

Fig.2: Identification of activated IFN-γ+ T lymphocytes after restimulation

Percentages of activated IFN-γ+CD4/CD8+ T lymphocytes measured by flowcytometry 24 h after restimulation in five independent experiments (P1-P5). The graph shows median, minimum and maximum values (%) and the statistical significance of differences between the percentage of T lymphocytes activated using DCs loaded with hTERT (p_1), MUC1 (p_2), apo ARH 77 (p_3), or all three antigens (p_{total}) compared with T lymphocytes activated using DCs loaded with HIV-1 nonapeptide (negative control). The analysis was carried out using the Mann-Whitney test.

control. The percentages of activated T lymphocytes for hTERT nonapeptide was 0.94– 1.44% (median 1,14%; p_1 = 0,009) and 0.79–1.42% (median 1,22%; p_1 = 0.009) for CD4+ and CD8+ T lymphocytes, respectively. The values for MUC1 nonapeptide were 0.87–1.72% (median 1.42%; p_2 = 0.009) for CD4+ T lymphocytes and 0.78–1.43% (median 1.09%; p_2 = 0.009) for CD8+ T lymphocytes. In the case of apo ARH 77, the percentages of activated cells were 1.0–1.74% (median 1.28%; p_3 = 0.009) and 0.95–1.30% (median 1.19%; p_3 = 0.009) for CD4+ and CD8+ T lymphocytes, respectively (Figure 2). Thus, all three tumor antigens significantly increased the proportion of activated T lymphocytes in comparison with the negative control (HIV-1-derived nonapeptide), with p_{total} = 0.001 for both CD4 and CD8+ T lymphocytes (Figure 2).

Immunomagnetic separation of T lymphocytes activated using tumor antigen-loaded dendritic cells . Immunomagnetic separation of T lymphocytes activated using DCs loaded with apo ARH 77 and hTERT or MUC1 nonapeptides was carried out on day 7 of culture. Figure 3 shows the percentages (median, minimum, and maximum values) of activated IFN-γ+ CD4/CD8+ T lymphocytes in the pre-separation fraction as well as in the positive and negative fractions after separation for each antigen separately. The immunomagnetic selection was performed twice in order to increase the purity of the positive fractions. It led to significant enrichment of activated T lymphocytes for all three antigens (i.e., apo ARH 77, and hTERT and MUC1 nonapeptides) to final percentages of 36.50–92.87 % (median 71.10%) for CD4+ and 29.40–94.08 % (median 70.98 %) for CD8+ T lymphocytes (p_{CD4} = 0.001 and p_{CD8} = 0.001 for comparisons with the pre-separation fraction) (Figure 3). The results of immunomagnetic separation for individual antigens in five independent experiments (E1-E5) are shown in Figure 3. The best enrichment was achieved for T lymphocytes activated with hTERT nonapeptide-loaded

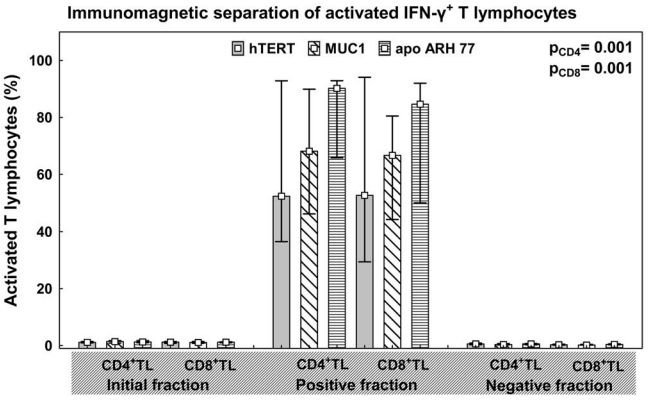

Fig.3: Immunomagnetic separation of activated IFN-γ+ T lymphocytes

The percentages of activated IFN-γ+CD4/CD8+ T lymphocytes stimulated using DCs with apo ARH 77, hTERT and MUC1 nonapeptides in the pre-separation fraction and after immunomagnetic selection for 5 independent experiments (positive and negative fractions are shown). The immunomagnetic separation was performed 24h after restimulation. Median, minimum, and maximum values are shown (%) as well as the statistical significance of IFN-γ+CD4+ (p_1) and IFN-γ+CD8+ (p_2) enrichment in the positive fraction compared to the pre-separation fraction. Data were analyzed using the Wilcoxon test.

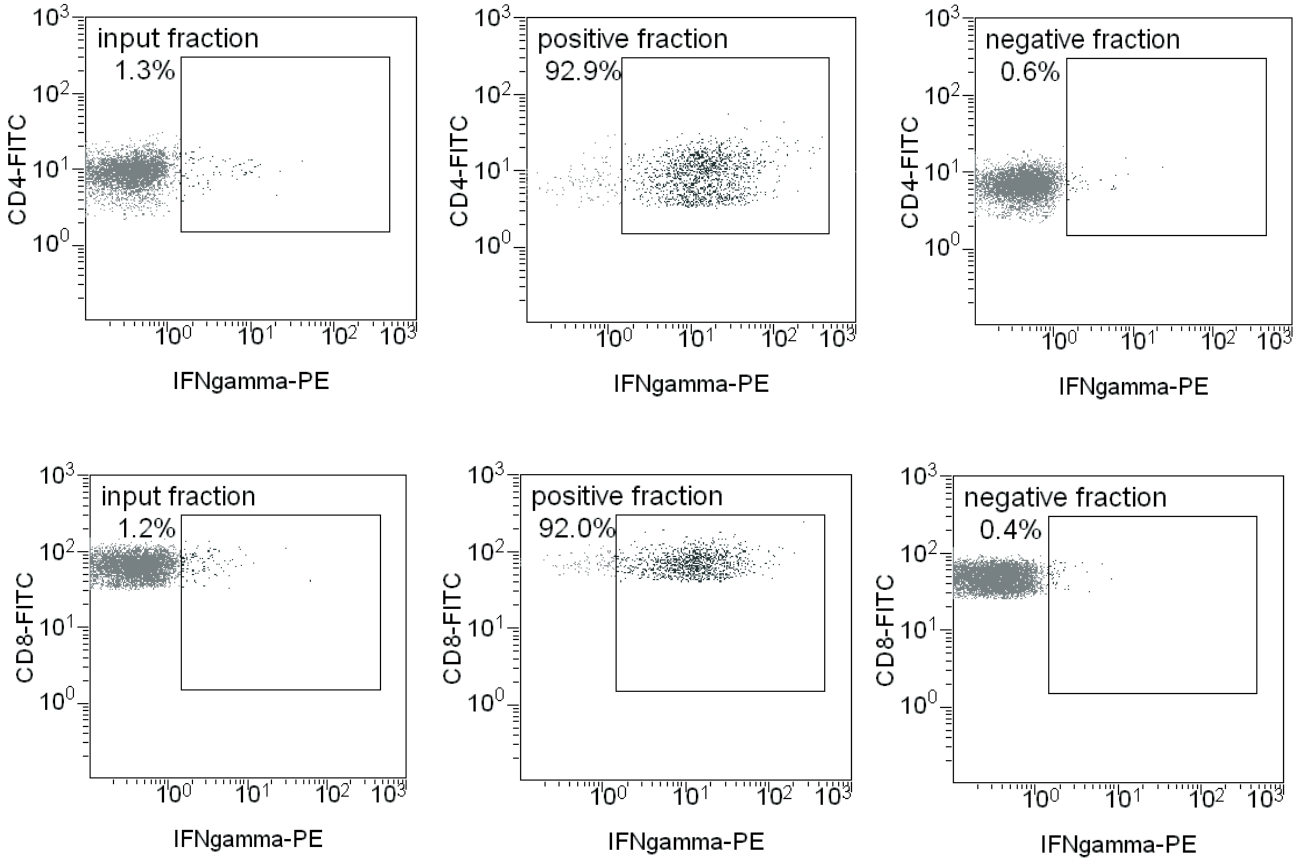

Fig. 4: Immunomagnetic separation of apo ARH 77 specific IFN-γ⁺ T lymphocytes

Enrichment of IFN-γ⁺CD3⁺CD4⁺ and IFN-γ⁺CD3⁺CD8⁺ apo ARH 77-specific T lymphocytes by immunomagnetic separation (results of a representative experiment for apo ARH 77 are shown.)

DCs (experiment E4) and for those activated with apo ARH 77-loaded DCs (experiment E5) (Figure 4).

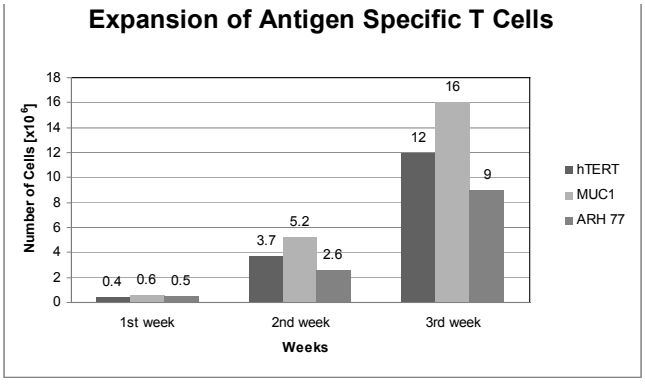

Fig.5: Expansion of Antigen Specific IFN-γ⁺ T Cells *in vitro*.

Median values for five independent experiments for each antigen are shown.

Expansion of immunomagnetically separated antigen-specific T lymphocytes . IFN-γ⁺ hTERT/MUC1/apo ARH 77 -specific T lymphocytes after immunomagnetic separation were expanded *in vitro* using paramagnetic microbeads conjugated with anti-CD3 and anti-CD28 antibodies and IL-2.

The number of cells seeded for expansion depended on the yield of immunomagnetic separation. We seeded 0.3–0.6 x 10^6 (median 0.4 x 10^6) hTERT-specific T lymphocytes, 0.30–0.8 x10^6 (median 0.5 x 10^6) MUC1-specific T lymphocytes, and 0.4–0.6 x10^6 (median 0.5 x 10^6) apo ARH 77 T lymphocytes.

The duration of expansion was 3 weeks. The number of expanded cells ranged from 8 to 16 x 10^6 T lymphocytes (median 12 x 10^6) for hTERT, from 10 to 20 x 10^6 T lymphocytes (median 16 x 10^6) for MUC1, and 5 to 13 x10^6 T lymphocytes (median 9 x 10^6) for apo ARH 77 T lymphocytes (Figure 5). Overall, the number of seeded T lymphocytes for all five independent experiments with each antigen was 0.3–0.8 x 10^6 T lymphocytes (median 0.5 x 10^6 cells) whereas the number of harvested T lymphocytes after expansion was 5–20 x 10^6 cells (median 12 x 10^6 cells).

Cytotoxicity assay. [$DiOC_{18}$ (3)] - labeled T lymphocytes were co-cultured with ARH 77 cells in effector:target (E:T) ratios of 20:1 and 40:1 for 4 hours. Expanded T lymphocytes from IFN-γ-positive fractions of all antigens (hTERT, MUC1 and apo ARH 77) exhibited statistically significant cytotoxic potential against ARH 77 myeloma cell line (Figure 6). Two negative controls were used: expanded T lymphocytes from the IFN-γ-positive fraction mixed with third-party allogeneic PBMCs, and ARH77 cells alone.

The effector: target cell ratio of 40:1 (T lymphocytes: ARH 77 myeloma cells) was optimal for cytotoxicity measurement, with the highest tumor cell-killing rate for all types of tested antigen-specific T lymphocytes (i.e., those directed against hTERT, MUC1, or apo ARH 77) (Figure 6).

Discussion

MM has been considered poorly immunogenic cancer. So far, immunotherapy based on tumor-specific vaccination or dendritic cell based approaches [53] has been associated only with limited success in MM patients. Recently, it has been demonstrated that CTL activated by specific tumor antigen and producing IFN-γ can be captured and used for targeted immunotherapy of cancer [54]. We have been developing this strategy further by demonstrating that activated IFN-γ-positive T cells can be expanded to large numbers without the loss of tumor specificity. Such approach can be used clinically even in poorly immunogenic diseases such as MM.

We have tested our approach in an allogeneic setting since the graft-versus-myeloma (GVM) effect has been well documented in clinical studies and survivors of allogeneic transplantations may experience fewer relapses than survivors of autologous transplantations [54, 55, 56]. The principal clinical problem with allogeneic hematopoietic cell transplantation in MM is severe graft-versus-host disease (GVHD) and opportunistic infections associated with high morbidity and mortality reaching 50% after standard myeloablative conditioning regimens [55]. Robust donor hematopoiesis can also be established using nonmyeloablative regimens with only transient myelosupression and minimum risk of consequent infection. Still 25-35% of patients after nonmyeloablative transplantation eventually succumb in the first year, mainly as a consequence of GHVD [56, 57]. Despite the fact that GVHD frequently accompanies GVM effect [55, 56], we were able to demonstrate previously that donor T cells leading to GHVD can be separated from graft-versus-tumor (GVT)-reactive T cells at least in leukemia patients [58]. We were also able to selectively remove the harmful GVHD-reactive T cell clones from a donor inoculum while GVT-reactive clones were preserved intact even in a clinical scale of the donor graft [58, 59]. Such graft engineering using an anti-CD25 immunotoxin for selective depletion of alloreactive T cells has already been used clinically and led to a significant and profound decrease of GVHD severity [60].

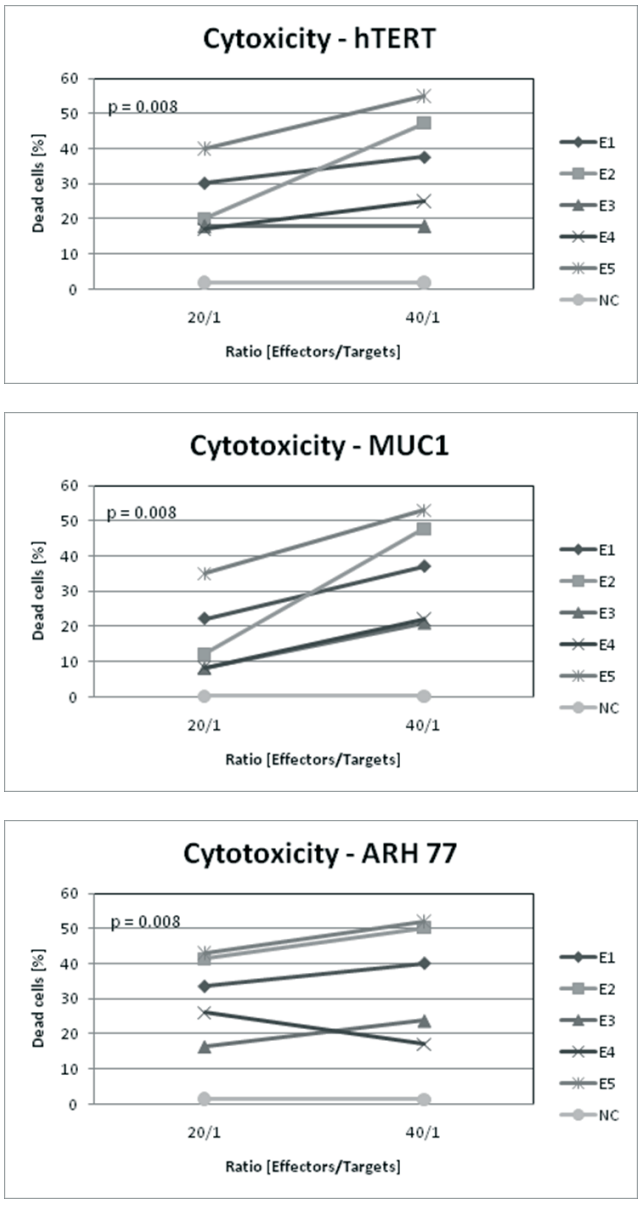

Fig.6: The cytotoxicity test of hTERT/MUC1/apo ARH 77 specific T Cells against myeloma cells

The graphs show the percentage of dead target cells (ARH 77 myeloma line cells) after 4h of co-culture with antigen-specific T lymphocytes that were used as effectors. Statistical significance of difference between the rate of tumor cell killing and the killing of allogeneic PBMCs that were used as negative control is shown for each type of antigen-specific T lymphocytes (against hTERT, MUC1, or apo ARH77 antigens). The cytotoxicity assay was carried out using two effector:target cell ratios of 1:20 and 1:40 (T lymphocytes/ARH 77 cells). Five experiments were performed for each type of antigen-specific T lymphocytes (E1-E5). Data were analyzed using the Mann-Whitney test.

In our present study, we have been able to demonstrate the possibility of myeloma-specific allogeneic T cell preparation using a relatively simple in vitro technique that may be useful

especially in slowly progressing malignancies such as MM allowing in vitro preparation and expansion of tumor-specific T cells. In allogeneic setting, the harmful GVHD-reactive cells can be selectively depleted by the anti-CD25 immunotoxin as suggested in the studies mentioned above [58, 59, 60]. Thus, this method permits the expansion of optimal anti-tumor T lymphocyte populations for an individual patient.

Other methods have been applied for the expansion of antigen-specific T lymphocytes, including selection and sorting using MHC tetramers or cloning by limiting dilution [54,61]. However, these methods have significant limitations. Isolation of CTL by tetramers requires knowledge of the donor's MHC as well as of tumor-associated antigens.

In conclusion, we have shown in this study that myeloma antigens such as hTERT, MUC1 and apo ARH 77 can induce myeloma-specific T cell responses *in vitro*. Repeated stimulation by these antigens leads to the activation of myeloma-reactive allogeneic T lymphocytes that are producing IFN-γ. T lymphocytes isolated on the basis of IFN-γ production can be further expanded to numbers suitable for a clinical-grade adoptive immunotherapy, retaining their specificity.

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