

In Vitro Activation of Cytotoxic T Lymphocytes By Muc1 Pulsed Dendritic Cells

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Abstract: Multiple myeloma (MM) is one of the most common hematologic malignancies. Malignant plasma cells are only weakly immunogenic and the disease is accompanied by a defective function of the immune system. An important issue for immunotherapy of myeloma is the identification of appropriate tumor-associated antigens. Recently, MUC1 was detected on a majority of myeloma cell lines. We studied antigen-specific and HLA-A2-restricted cytotoxic activity against MUC1-positive ARH77 myeloma cell line *in vitro*. A HLA-A2 specific MUC1-derived nonapeptide (¹³⁹TSAPDTRPA¹⁴⁷) was used as a tumor-associated antigen. Myeloma-specific cytotoxic activity of MUC1-reactive CTL was established by repeated stimulation of CTL via dendritic cells loaded with MUC1-derived nonapeptide. We were able to demonstrate that MUC1-reactive T cells can be identified and expanded using a relatively simple *in vitro* techniques. Antigen-specific stimulation via dendritic cells, immunomagnetic sorting and rapid expansion of CTL without losing of their specific immune activity can be used clinically even in poorly immunogenic diseases such as MM.

INTRODUCTION

Multiple myeloma (MM) is a hematologic cancer arising from post-germinal matured B cells characterized by an excess of monoclonic plasma cells in the bone marrow secreting monoclonal immunoglobulins with a concomitant decrease in normal immunoglobulins and lytic bone lesions [1]. Multiple myeloma has been considered a cancer with weak immunogenicity of the malignant cell population and defective function of the immune system [2, 3]. The identification of tumor-associated antigens has opened new opportunities for the immunotherapy of MM patients.

MUC1 is a highly glycosylated type I transmembrane glycoprotein with an extracellular domain consisting of 20-60 tandem repeats [4]. Its expression on normal cells is hidden from the immune system, and its aberrant glycosylation creates new epitopes on tumor cells that can be recognized by the immune system [5]. MUC1 is overexpressed in breast, ovarian, renal and pancreatic cancers [6-8]. MUC1 was also identified in the serum and the bone marrow of MM patients [9-11] as well as in most myeloma cell lines [12, 13]. It is a recognized tumor-associated antigen (TAA) and has been proposed as a candidate for anti-tumor vaccination [6, 7].

Vaccinations using dendritic cells pulsed with different TAAs were shown to be effective in patients with different cancer types including MM, B-cell lymphoma and malignant melanoma [14-18]. Dendritic cells (DCs) are the most potent

antigen-presenting cells (APC) with a unique ability to initiate primary immune responses of T cells [19-24]. Pilot studies showed that the application of dendritic cells loaded with MUC1 nonapeptide was able to induce immune responses in patients with breast and ovarian cancer [6]. Recently, it has been demonstrated that autologous mature dendritic cells pulsed with HLA-A2-binding MUC1 peptides caused regression of metastatic sites in renal cell carcinoma patients [7]. MUC1-specific CD8⁺ cytotoxic T lymphocyte (CTL) response has also been detected in HLA-A2-positive myeloma patients [10].

In this study, we analyzed the antigen-specific HLA-A2-restricted cytotoxic activity against ARH77 myeloma cell line *in vitro* using HLA-A2-specific MUC1-derived nonapeptide (¹³⁹TSAPDTRPA¹⁴⁷) as a TAA. Activation of CTL was performed via MUC1-pulsed dendritic cells.

MATERIALS AND METHODS

Cell Isolation and Cultures

Peripheral blood was collected from HLA-A2 positive healthy volunteers after signing the informed consent approved by the local Ethical Committee. Peripheral blood mononuclear cells (PBMCs) were isolated with Histopaque 1077 (Sigma-Aldrich, Prague, Czech Republic) density gradient centrifugation from buffy coats of healthy donors. Isolated PBMC were cultivated in X-VIVO 10 (BioWhittaker, Walkersville, MD, USA) supplementing with 10% human AB-serum (Sigma-Aldrich, Prague, Czech Republic), 80U/ml DNase (Boehringer, Mannheim, Germany) and 1mM L-glutamine (Sigma-Aldrich, Prague, Czech Republic). After 2-hours of incubation at 37°C incubator with 5%

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CO₂ cells were separated to adherent and non-adherent fractions. Non-adherent fraction that was rich of T lymphocytes was incubated in complete medium (CM) containing X-VIVO 15 (BioWhittaker, Walkersville, MD, USA), 10% human AB-serum (Sigma-Aldrich, Praha, Czech Republic) and 10 IU/ml IL-2 (Proleukin, Chiron, Amsterdam, Netherlands). 10 IU/ml IL-2 was added every 2 to 3 days.

Adherent fraction with DC precursors was cultured for 6 days in X-VIVO 10 (BioWhittaker, Walkersville, MD, USA) supplemented with 20 ng/ml IL-4 (CellGenix, Freiburg, Germany) and 100 U/ml GM-CSF (CellGenix, Freiburg, Germany). DC medium with cytokines was replaced every 2 days and cell morphology was monitored by light microscope [17, 18]. After 6 days of DC culture, maturation agents 10 ng/ml TNF- α (Bender Medsystems Diagnostics, Vienna, Austria), 10 ng/ml IL-1 β (CellGenix, Freiburg, Germany), 250 ng/ml PgE₂ (Prostin, Pfizer Inc., NY, USA), 1 μ g/ml TLR-ligand R848 (Invivogen, California, USA) and 5000 IU/ml IFN- γ (Gentaur, Brussels, Belgium) were added [25-28]. Cells were harvested after overnight incubation.

Peptide Antigens

HLA-A2 specific MUC-1-derived synthetic peptide (amino acids ¹³⁹TSAPDTRPA¹⁴⁷) (Proimmune, Oxford, UK) was used as a TAA. An HLA-A2 binding HIV-1 reverse transcriptase synthetic peptide (amino acids ⁴⁷⁶ILKEP VHG⁴⁸⁴) was used as a negative control (Proimmune, Oxford, UK) [6, 28]. Matured dendritic cells were pulsed for two hours with 1 μ g/ml peptide on day 7 of DC culture as previously described [6].

Stimulation and Restimulation of T Lymphocytes

On day 7 peptide-pulsed DCs were mixed with T lymphocytes at the ratio 20:1 (T cells:DCs). The remaining DCs were frozen and used for T lymphocyte restimulation at the ratio 2:1 (T cells:DCs) after 7 days following the initial stimulation as previously described [29].

IFN- γ Detection and Immunomagnetic Separation

The IFN- γ production was performed with Secretion Assay Cell Enrichment and Detection Kit (MACS Reagents, Miltenyi Biotec, Bergisch Gladbach, Germany).

Cell separation was performed 24 hours after restimulation of T cells with peptide-pulsed DCs (MUC1 or pol HIV-1). Following the manufacturer's instructions, immunomagnetic beads were used for sorting with VarioMACS cell separation system (MACS Reagents, Miltenyi Biotec, Bergisch Gladbach, German). IFN- γ ⁺ T lymphocytes were labeled with paramagnetic beads from Secretion Assay Cell Enrichment and Detection Kit (MACS Reagents, Miltenyi Biotec, Bergisch Gladbach, Germany) [28]. All solutions were kept on ice to prevent capping and patching of antibodies on the surface of labeled cells. To increase the purity, IFN- γ ⁺-fraction containing antigen-reactive T cells was separated twice. IFN- γ ⁺ fraction was then expanded *in vitro*. Cell purity was checked by flow cytometry.

Flow Cytometry

Cells were stained using fluorescein isothiocyanate (FITC)-, phycoerythrin-cyanine 7 (PC7)- or phycoerythrin

(PE)-conjugated monoclonal antibodies against CD4, CD8, CD3, CD 80, CD 86, CD 83, HLA DR (all purchased from Immunotech, Marseille, France) and IFN- γ (Miltenyi Biotec, Bergisch Gladbach, German). Flow cytometry analyses were performed with Cytomics™ FC 500 (Beckman Coulter, Miami, Florida, USA).

Expansion of IFN- γ ⁺ MUC1-reactive T lymphocytes

IFN- γ ⁺ MUC1-reactive T lymphocytes were expanded *in vitro* with 75 μ g/ml of anti-CD3/anti-CD28 monoclonal antibodies (Dynabeads CD3/CD28 T cell Expander, Invitrogen, California, USA). Cultured cells were plated (50 \times 10³ T cells per well) into 96-well plates in complete medium supplemented with 500 IU/ml interleukin-2 (Proleukin, Chiron, Amsterdam, Netherlands). Cultures were fed with fresh medium as needed and IL-2 was added twice a week. Irradiated allogenic PBMCs (30 Gy) were added weekly.

Cytotoxicity Assay

T cell-mediated cytotoxicity assay was performed with the LIVE/DEAD[®] Cell-Mediated Cytotoxicity Kit (Invitrogen Corporation, Carlsbad, USA). MUC1-positive ARH77 myeloma cell line was used as a target and was labeled with 3,3'-diiodoacetylcarboxyanine (DiOC) as previously described [31]. To test their specificity, MUC1-reactive T cells (effector cells) were mixed with target cells at different ratios and incubated for 4 hours. Dead target cells were indicated with propidium iodide (PI) staining [54]. Maximum target cell lysis was measured after irradiation (60 Gy) of ARH77 cells and spontaneous target cell lysis was measured after incubation without effectors. As a negative control, third-party PBMCs were cultivated with MUC1-specific CTL. The percentage of cell-mediated cytotoxicity was calculated by the following formula: cytotoxicity (%) = (experimental target cell lysis - spontaneous target cell lysis)/(maximum target cell lysis - spontaneous target cell lysis) \times 100.

Statistical Analysis

Statistical analysis was performed with software package SPSS 16.0 ([©] SPSS Inc.). Results were submitted with descriptive statistics and were reported as Mean \pm SD. Significance was estimated by nonparametric tests. Two independent sample analyses were performed with Mann-Whitney U test. Wilcoxon signed-rank test was used to compare the distributions of two related variables.

RESULTS

Activation of T lymphocytes by MUC1-pulsed Dendritic Cells

DCs were generated from PBMCs using GM-CSF and IL-4 and were activated by addition of TNF α as previously described. Maturation of DCs with TNF α led to high expression of CD80, CD86, HLA-DR (\geq 85%) and at least intermediate expression of CD83 (\geq 25%) molecules. Matured DCs were loaded with nonapeptide antigen (¹³⁹TSAPDTRPA¹⁴⁷) derived from MUC1 protein. MUC1-pulsed DCs served as stimulators of autologous CD4⁺ and CD8⁺ T lymphocytes. After repeated stimulation of T cells, the IFN- γ ⁺ subset of CD3⁺CD4⁺ and CD3⁺CD8⁺ reached 1.31 \pm 0.15% and 1.05 \pm 0.12%, respectively. In the control group with HIV-1 peptide, the IFN- γ ⁺ subset of CD3⁺CD4⁺ and CD3⁺CD8⁺ was

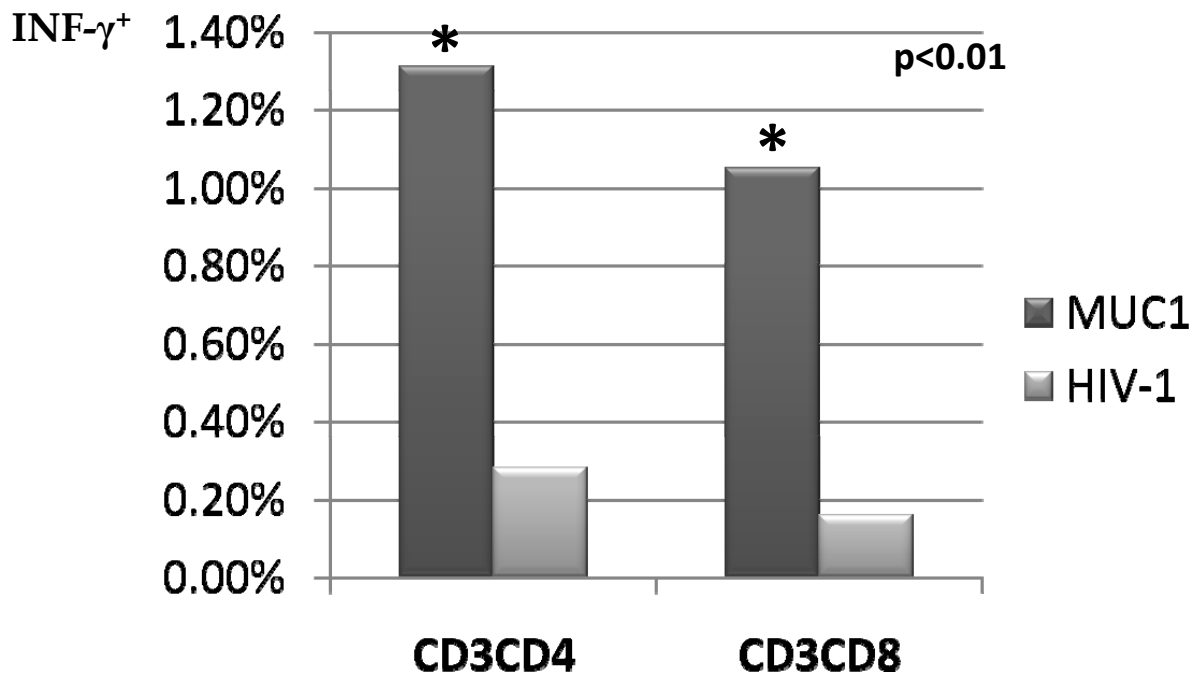


Fig. (1). IFN- γ production of MUC1-reactive T cells.

Matured dendritic cells were loaded with nonapeptide antigen ($^{139}\text{TSAPDTRPA}^{147}$) derived from the MUC1 protein and used to simulate autologous T cells. After 7 days following the initial stimulation, T lymphocytes were restimulated with the same antigen-pulsed DCs. HIV-1-derived peptide ($^{476}\text{ILKEPVHGV}^{484}$) was used as negative control. Activation of $\text{CD3}^+\text{CD4}^+$ and $\text{CD3}^+\text{CD8}^+$ T lymphocytes in the experimental (MUC1) and the control (HIV-1) groups was measured by the IFN- γ production. Flow cytometry analysis was performed after 24-hours of restimulation with MUC1- or HIV-1-pulsed DCs. All data were shown in the text.

$0.28\% \pm 0.019\%$ and $0.16\% \pm 0.037\%$, respectively. The differences between experimental and control groups were significant ($p < 0.01$) (Fig. 1).

Immunomagnetic Separation of MUC1-reactive T lymphocytes

To enrich the MUC1-reactive population of T cells, the IFN- γ^+ T cells were separated in the magnetic field. Pre-purification IFN- γ^+ subset of $\text{CD3}^+\text{CD4}^+$ and $\text{CD3}^+\text{CD8}^+$ T cells was $1.31\% \pm 0.15\%$ and $1.05\% \pm 0.12\%$, respectively. To increase the purity of MUC1-reactive T cells the IFN- γ^+ fraction was separated twice. After double separation the MUC1-reactive T cells were enriched to $66.94\% \pm 7.19\%$ of IFN- γ^+ $\text{CD3}^+\text{CD4}^+$ and $64.59\% \pm 6.03\%$ IFN- γ^+ $\text{CD3}^+\text{CD8}^+$ T cells. The negative fraction contained less than 0.5% of IFN- γ^+ T cells after double immunomagnetic separation (Fig. 2).

Expansion of IFN- γ^+ MUC1-Reactive T lymphocytes

Expansion of MUC1-reactive IFN- γ^+ T lymphocytes after immunomagnetic separation was started with $0.1\text{-}0.3 \times 10^6$ T cells. In all experiments T cells were successfully expanded to at least 1.0×10^7 T cells within 8-12 days with anti-CD3/anti-CD28 beads.

Cytotoxicity Assay

To confirm the specificity of MUC1-reactive T cells, the cytotoxicity test with ARH77 MUC1-positive myeloma cell line as target cells was performed. Expanded MUC1-reactive T cells were used as effectors. Specific cytotoxicity of MUC1-reactive T cells was measured with flow cytometry-based test. At a ratio 20:1 (effector:target cells) cytotoxicity

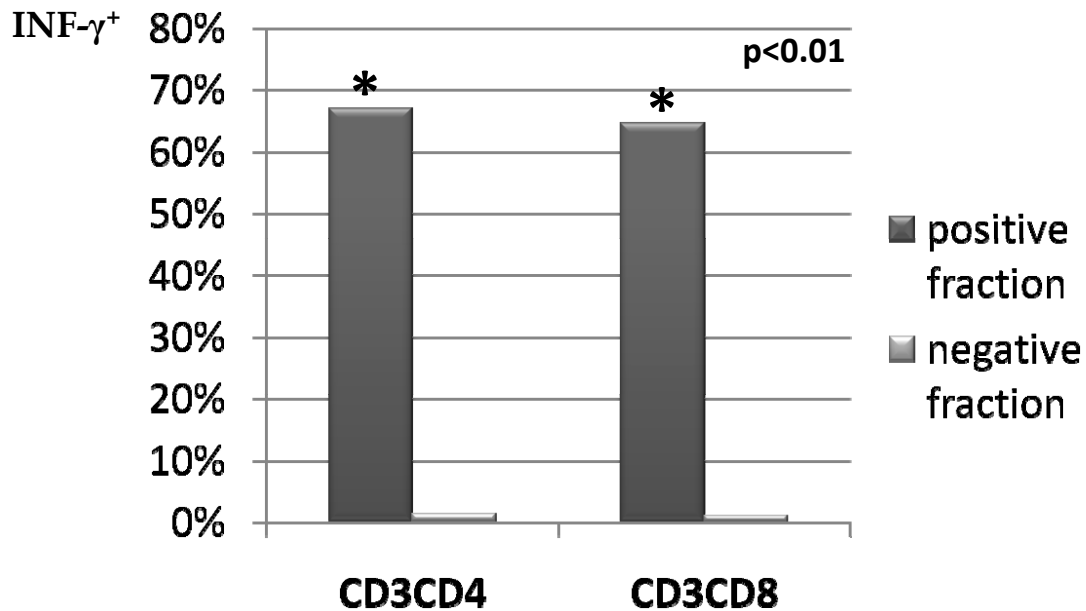
of IFN- γ^+ T lymphocytes vs. ARH77 reached $17.16\% \pm 5.13\%$ compared to non-specific killing of allogenic PBMC which was $0.79\% \pm 0.39\%$. At a ratio 40:1 IFN- γ^+ T cells were able to kill $36.14\% \pm 6.51\%$ of ARH77 MUC1-positive target cells while the cytotoxicity against allogenic PBMC remained negligible $0.77\% \pm 0.38\%$ (Fig. 3). The data demonstrate a specific cytotoxic potential of expanded MUC1-reactive T cells that are able to recognize MUC1 and kill target cells carrying that antigen.

DISCUSSION

Multiple myeloma has been considered as a cancer with weak immunogenicity of the malignant cell population and a defective function of the immune system, including dendritic cells and T cells [2, 3]. MUC1-specific CD8^+ T cells response was recognized in 40% of HLA-A2-positive myeloma patients [10]. MUC1 expression was found in 92% of myeloma samples [36]. Based on our previous observations [29, 30] and other studies [5, 6, 10, 11] we were able to demonstrate that MUC1-pulsed DCs that are not immunocompromised by the original cancer environment can induce myeloma-specific CTLs. Functional DCs are critical for optimal antigen presentation and for initiation of T-lymphocyte response. Significant increase of T-lymphocyte IFN- γ production confirmed immunogenic activity of MUC1-pulsed DCs.

CD8^+ T cells can differentiate into cytotoxic TLs which are able to lyse TAA-expressing cells. Activation of CD8^+ T cells requires two steps: presentation of antigenic peptides on professional APC and helper function which is provided by CD4^+ T cells [32-36]. In our study anti-tumor activity of

A.



B.

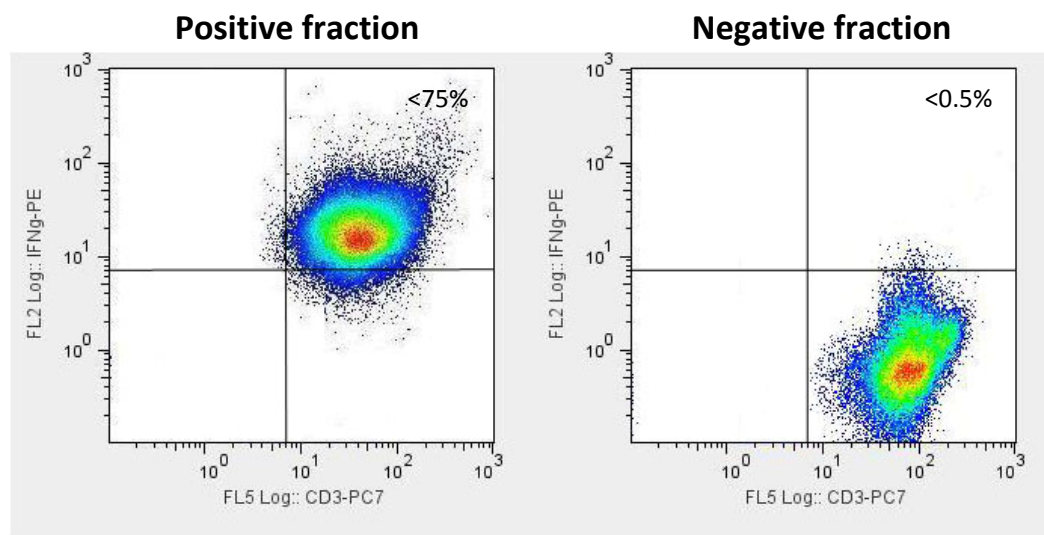


Fig. (2). Separation of MUC1-reactive T cells.

Comparison of IFN- γ^+ subsets of CD3 $^+$ CD4 $^+$ and CD3 $^+$ CD8 $^+$ T lymphocytes in positive and negative fractions. Immunomagnetic separation was performed twice. Panel A demonstrates percentages of IFN- γ producing cells of each subset. Panel B demonstrates staining of gated lymphocytes with PE-conjugated anti-IFN- γ and PC7-conjugated anti-CD3 from representative experiment. All data were shown in the text.

MUC1-specific IFN- γ^+ T cells was evaluated *in vitro*. The cytotoxicity test was used as an integrative indicator of anti-tumor immune response. MUC1-specificity of cytotoxic attack was associated with MUC1 expression by the myeloma cell-line ARH77.

Recently, it has been demonstrated that cytotoxic T lymphocytes activated by specific tumor antigen and producing IFN- γ can be captured and used for targeted immunotherapy of cancer. In our previous study we adapted this strategy further to demonstrate that activated IFN- γ^+ T cells can be expanded to large numbers without loss of tumor specificity using PHA [29]. PHA serves as a potent stimulus for T cell expansion but, it has been also demonstrated that long-term

repeated PHA stimulation of T cells may lead to their exhaustion [37]. Thus, short term PHA stimulation or other strategies that employ anti-CD3/CD28 beads can be used for rapid clinical-grade T cell expansion [38]. In this study we were able to demonstrate the possibility rapid and efficient CTL expansion technology without losing of their specific immune activity. Anti-CD3/CD28 based expansion of CTLs can be an important ingredient of successful CTL-targeted therapy.

CONCLUSION

In this study, we were able to demonstrate that MUC1-specific T cells can be identified and expanded using a rela-

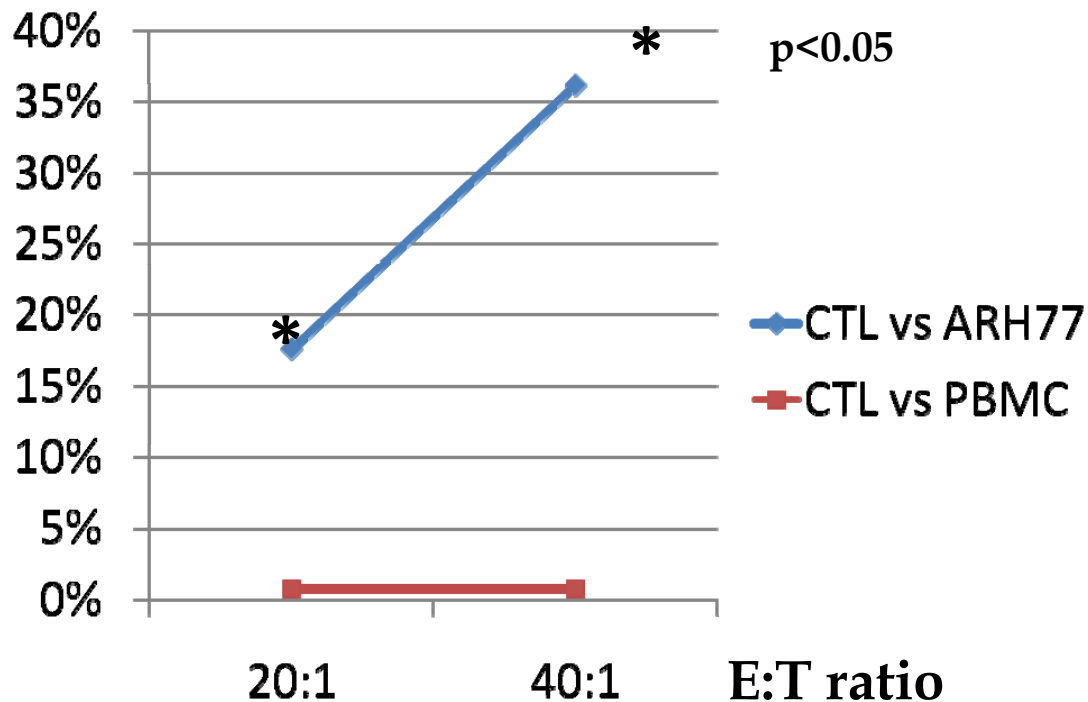


Fig. (3). Cytotoxic activity of MUC1-specific CTL against ARH77 myeloma cell line.

T cell-mediated cytotoxicity was performed with expanded MUC1-reactive T cells as effectors (E). DiOC-labeled MUC1-positive ARH77 myeloma cells were used as target cell (T). PBMCs were used as a negative control target. All data were shown in the text.

tively simple *in vitro* technique consisting of antigen-specific stimulation via DC, immunomagnetic sorting and rapid expansion. Such approach can be used clinically even in poorly immunogenic diseases such as MM. In summary, MUC1-pulsed DCs and MUC1-specific CTL could be good candidates for future clinical trials. In case of multiple myeloma the MUC1-specific vaccination (DC- or CTL-based) can be a great benefit leading to alert the immune system against such tumor antigen and possible myeloma progression.

ACKNOWLEDGMENT

This study was supported with grant MSMT LC06027 and IGA MZCR 1A8709-5.

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Received: March 16, 2009

Revised: August 14, 2009

Accepted: September 12, 2009

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