Low antigen-dependent activity of T cells after repeated stimulation using dendritic cells and expansion with interleukin-2*

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Both CD8+ and CD4+ T cells with specific activity against tumor antigens are needed for an efficient antitumor immune response. Activation and proliferation of T cells require cellular interactions including adhesion, recognition of peptides presented by MHC molecules to the T cells receptor, and costimulation. In a series of experiments we attempted to generate and expand specific T cells by repeated stimulation using antigen-loaded autologous dendritic cells (DCs). DCs were obtained from peripheral blood mononuclear cells (PBMC) in the presence of IL-4 and GM-CSF. TNF-α was added to induce maturation. A conjugate of myeloma idiotypic protein with keyhole limpet hemocyanin was used as antigen. Nonadherent peripheral blood mononuclear cells were cultured in the presence of Il-2 and IL-7. Autologous DCs were added to the lymphocyte cultures on days 3, 10, and 17. The lymphocytes were stimulated by high concentration of IL-2 between days 21 and 27. Lymphocytes harvested on day 27 proliferated in response to antigen-loaded DC but failed to do so if less than $0.3x10^6$ DCs were added for stimulation during culture. However, no cytotoxic activity against autologous DCs was detected and IFN-γ production in the T cell cultures was low at the end of culture. In conclusion, the generation and expansion of T cells using repeated stimulation by autologous DCs is feasible but defective cytotoxic reponse of these cells occurs, possibly as a consequence of repeated frequent exposure to antigen.

Key words: T cells, dendritic cells, immunotherapy, in vitro cell culture.

Both CD8+ and CD4+ T cells with specific activity against tumor antigens are needed for an efficient antitumor immune response. Cytotoxic T lymphocytes (CTLs) are responsible for the lysis of tumor cells, while CD4+ cells provide some vital costimulatory signals [5, 9, 16]. Methods for specific antitumor CTL production and expansion are rapidly developing and may soon be available for applications in several types of cancer enhancing the efficacy of current immunotherapeutic protocols [2].

Activation and proliferation of T-cells require cellular interactions including adhesion, recognition of peptides presented by major histocompatibility complex (MHC) molecules to the T-cell receptor and costimulation. Costimulation is neither antigen-specific nor MHC-restricted and can be mediated via a large number of cell surface molecules expressed on antigen-presenting cells [6].

Dendritic cells (DCs), the most effective antigen-presenting cells known, play a key role in the induction of CD8 and CD4 responses *in vivo* and *in vitro*. They exhibit specialized phagocytic activity and antigen capture by receptor-mediated uptake and macropinocytosis, ability to process and present an antigen within the MHC I and II, and express a variety of costimulatory molecules that are critical for the induction of primary CTL responses [1, 3, 4, 7].

Multiple stimulations with antigen-presenting cells are thought to be necessary for an efficient production and expansion of autologous tumor-specific CTLs [15, 22]. However, repeated engagement of the T-cell receptor by antigen

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in the absence of costimulation fails to induce T-cell activation but rather results in the induction of long-term unresponsiveness – anergy [20, 21].

In our study we attempted to generate and expand T cells directed against a model antigen – a keyhole limpet hemocyanin conjugated with myeloma idiotypic (Id) protein – by repeated *in vitro* stimulation of lymphocytes using antigen-loaded autologous dendritic cells.

Material and methods

Cell cultures. Peripheral blood mononuclear cells (PBMCs) from venous blood of three healthy volunteers were prepared by standard gradient centrifugation using Histopaque (Sigma Chemicals, St Louis, MO, USA). The culture medium consisted of X-VIVO 10 (Bio Whittaker, Walkersville, MD, USA) with 2% human albumin (USOL, Prague, Czech Republic), 80 U/ml DNAse (Boehringer Mannheim, Germany), and 1 mM glutamin (Sigma Chemicals). The cells were plated in six 3-ml wells at 37 ˚C in the atmosphere of 5% CO_2 and 4.5% O_2. Taking advantage of adherence of DC precursors after 2-hour culture, nonadherent cells were discarded with the supernatant as described [17]. Adherent cells were cultured after adding 100 ng/ml granulocyte and macrophage colony-stimulating factor (GM-CSF, Schering Plough, Kenilworth, NJ, USA) and 100 ng/ml interleukin (IL)-4 (Sigma Chemicals). On days 3 and 6, 50% of the medium was changed with the addition of cytokines, glutamine and albumin. The antigen prepared as described below was added on day 5 in a quantity of 50 μg/ml. Maturation was induced by the addition of 1000 U/ml tumor necrosis factor (TNF)-α (Bender Medsystems Diagnostics, Vienna, Austria) on day 7. On day 9, the cells were harvested and added to the T cell culture.

For T cell culture, nonadherent PBMCs cells were suspended in a RPMI 1640 medium (Sigma Chemicals) with 5000 UI/ml heparin (Leciva, Prague, Czech Republic), 2% human serum (Sigma Chemicals), 5000 UI/ml penicilin (USOL), 5 mg/ml streptomycin (USOL), 0.4 mol/l L-glutamin (Sigma Chemicals), 10 U/ml IL-2 (Chiron, Amsterdam, The Netherlands), and 10 ng/ml IL-7 (Biosource, Camarillo, CA, USA). Every two days, 50% of the culture medium was changed and the cytokines added. On days 21 to 27 the concentration of IL-2 in the culture medium was increased to 40 U/ml. Autologous antigen-loaded dendritic cells were added on days 3, 10, and 17.

Antigen preparation. Id-protein isolation and its conjugation with keyhole limpet hemocyanin (KLH) was performed as described previously [10]. For purification of IgG myeloma proteins, plasma proteins were precipitated using ammonium sulfate. The samples were stirred overnight at 4 ˚C and then centrifuged at 12,000 g for 20 min. The pellet was redissolved and dialyzed against normal saline. The suspension was passed through a column containing protein G immobilized on agarose (Sigma Chemicals). For conjugation, KLH (Sigma Chemicals) was added to a weight ratio of 1:1 in the presence of glutaraldehyde (5%). The conjugate was then mixed with Polymyxin B (Sigma Chemicals), incubated for 12 hours and centrifuged at 1500g for 5 min. The supernatant was dialyzed against normal saline overnight at room temperature and filtered though a 22 μm membrane filter.

Immunophenotyping. Samples for DC immunophenotyping were taken on days 0, 6, and 9. Immunophenotyping was performed using fluorescence-marked mononuclear antibodies: CD1a, CD11c, CD54, CD86, CD83, HLA-DR (Caltag, Burlingame, CA, USA). The antigens CD83, HLA-DR, and CD86 were determined as a combination. Cell yield 10 days of culture based on the expression of the CD83 antigen. Immunophenotyping of T cells was performed on days 0, 22, and 27. The following antigens and antigen combinations were determined: CD3, CD4, CD8, CD4/CD8, 45 RA, 45 RO, 3/45RA, 3/45RO, CD25, CD69 (Caltag).

Proliferation assay. Alamar Blue test (Biosource) was used to determine the proliferation of restimulated T cells in response to antigen presented by DCs according to the manufacturer's instructions. The lymphocytes ($1x10^5$ cells per well) in a RPMI 1640 medium with heparin 5000 UI/ml, 2% human serum, 5000 UI/ml penicilin, 5 mg/ml streptomycin, 0.4 mol/l L-glutamin, and 10 U/ml IL-2, were incubated for 10 hours with irradiated (30 Gy) PBMC-derived dendritic cells expressing the antigen in increasing quantities up to $1x10^5$ cells/well. The absorbances at 550 and 630 nm were measured using ELISA Microplate Reader (Dynatech, Alexandria, VA, USA). The difference between absorbances at these wave lengths corresponds to cell growth as the Alamar Blue indicator is converted to its redox form.

ELISA for interferon-γ, IL-12, and IL-10. Enzyme-linked immunosorbent assay (ELISA) was used for the assessment of cytokine content in T cell and DC cultures. Interferon-γ (IFN-γ) content in the T cell culture supernatants was assessed on days 0, 7, 10, 20, and 28 using the IFNγ ELISA Kit (Diaclone, Besancon, France) according to the manufacturer's instructions. Briefly, 100 μl of supernatant from T cell cultures was added into anti-IFN-γ antibody-coated plates followed by 100 μl of biotinylated anti-IFN-γ antibody. After 2-hour incubation the plates were washed, and incubated with streptavidin-horseradish peroxidase (100 μl) for further 30 minutes. The reaction was evaluated following the addition of a chromogen at an absorbance of 450 nm. IL-12 and IL-10 produced by DCs were measured on days 0, 7, and 9 using the IL-12 p70 ELISA Kit (Diaclone) and the IL-10 ELISA Kit (Biosource). The procedure was similar to that described for IFN-gamma.

Cytotoxicity assay. Standard ^{51}Cr release assay was performed as described previously using antigen-loaded auto-

logous DCs as targets and cells from lymphocyte cultures as effector cells [22]. Briefly, $5x10^6$ target cells (CD83+ DCs) were marked with 4 MBq of ^{51}Cr, rinsed twice, and plated with effector cells in ratios 1:10 to 1:50. After 4-hour incubation the supernatant was collected and the radioactivity counted in a gamma counter.

Statistical analysis. Descriptive statistics (arithmetic mean, median, range, standard deviation, standard error of the mean, percentile ranges) was used do describe various groups of data. If necessary, data were corrected by subtracting the background values. All calculated values are presented as mean standard error of the mean unless indicated otherwise.

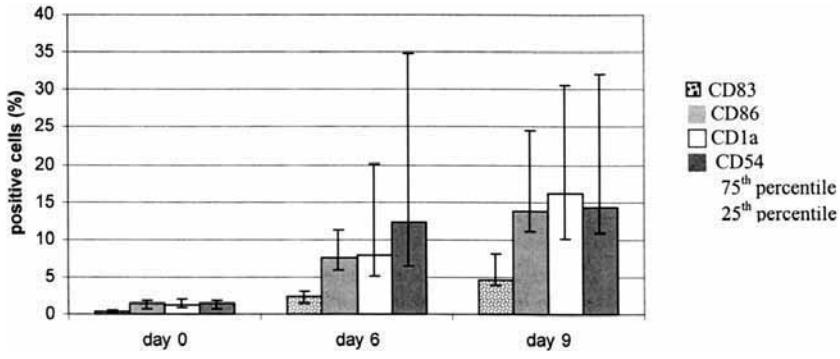

Figure 1. Percentages of cells expressing selected dendritic cell (DC) markers during 13 consecutive DC cultures. Adherent PBMC from venous blood were used as cell source.

Results

Generation of antigen-loaded, mature DCs from PBMCs. Adherent PBMCs from 60 ml of peripheral blood were grown for 10 days in culture medium supplemented with IL-4 and GM-CSF. Antigen (50 μg of Id-KLH conjugate) was added on day 5. DC maturation was induced by TNF-α on day 7. Bacterial cultures and testing for mycoplasma did not reveal any contamination in the 13 successive DC cultures. The mean yield of cells on day 9 of culture was $14.1 \pm 6.1x10^6$ cells per culture (75.3% HLA DR+ cells), $13.0 \pm 2.6x10^6$ cells per culture (90.2% HLA DR+ cells), and $6.0 \pm 0.4x10^6$ cells per culture (61.6% HLA DR+ cells) for donors 1, 2, and 3, respectively. The expression of selected DC markers is shown in Figure 1. The cells expressed mature DC phenotype. The mean yields of CD83+ cells which we believe are the most important for T cell response induction were $1.0 \pm 0.4x10^6$ cells per culture, $0.93 \pm 0.2x10^6$ cells per culture, and $0.22 \pm 0.1x10^6$ cells per culture for donors 1, 2, and 3, respectively. The CD83+ cell counts did not exceed $0.4x10^6$ cells in donor 3 in any of the five consecutive cultures.

IL-12 and IL-10 production in DC cultures. IL-12 and IL-10 were measured in supernatants from DC cultures. The presence of these cytokines is considered important in skewing the T-cell response toward Th1 or Th2 polarity, respectively. We detected about 70-fold increase in IL-12 concentration after 10-day culture which is consistent with DC activation as described previously [14]. The median value of IL-12 on day 9 was 94.7 pg/ml. During the same period, IL-10 levels fell to about 25% of the initial values and were below the detection limit in 8 of 13 DC cultures. IL-10 is a powerful immunosuppressor and low levels of the cytokine are desired for the optimal induction of T cell responses [15].

Evolution of cell counts and immunophenotype in lymphocyte cultures. Nonadherent cells from 60 ml of peripheral blood were used to set up the lymphocyte cultures. The PBMC count on day 0 ranged from $2.5–3.2x10^7$ cells per culture and expanded to $7.1–21.4x10^7$ cells per culture on day 27. Percentage of cells expressing selected lymphocyte antigens is shown in Table 1. The ratio of CD8 to CD4 cells increased during the first 22 days of culture in donor 1 but decreased in donors 2 and 3. Decreases in CD8/CD4 ratio as well as in CD3+45RA+ and CD25+ cells were seen in cells from all donors between day 22 and 27 when high-dose IL-2 was added to the lymphocyte culture. At the same time IFN-γ levels in the supernatant decreased sharply (see below). These results may reflect the inactivation of T cells because the cell counts remained stable or increased.

Table 1. Expression of selected T cell markers by cells in the T cell culture on day 0, 22, and 27 of culture

Antigen	Percentage of positive cells (range) day 0	day 22	day 27
CD3+	72.0 (58.6–83.2)	80.2 (57.8–92.5)	83.2 (73.5–89.9)
CD4+	42.8 (33.4–51.6)	41.4 (18.8–52.8)	49.0 (21.1–67.2)
CD8+	24.3 (21.9–30.7)	36.0 (12.4–67.7)	32.4 (9.1–68.6)
CD3+45RA+	38.6 (32.6–45.0)	18.3 (3.8–39.9)	16.0 (4.4–28.3)
CD3+45RO+	18.6 (14.4–21.2)	52.1 (38.4–71.5)	44.8 (27.4–54.7)
CD25+	1.0 (0.5–1.6)	15.8 (5.5–35.3)	5.7 (2.9–7.1)
CD69+	1.4 (0.8–2.45)	5.1 (1.7–10.7)	5.3 (2.0–8.9)

The decreases in CD3+CD45RA+, CD3+CD45RO+ and CD25+ cell content are evident. The values are representative of three cell cultures.

IFN-γ production after repeated stimulation of T cells. Supernatants from T cell cultures were taken on days 7, 10, 20, and 28, while antigen-presenting DCs were added on days 4, 10, and 20 of each T cell culture. The evolution of IFN-γ content in cell cultures is shown in Figure 2. After day 7, the levels of IFN-γ decreased in the cell culture supernatants. The upswing in IFN-γ concentration was not detected in cells from donor 3 which were stimulated by low doses of CD83+ cells (less than $0.3x10^6$ cells per stimulation).

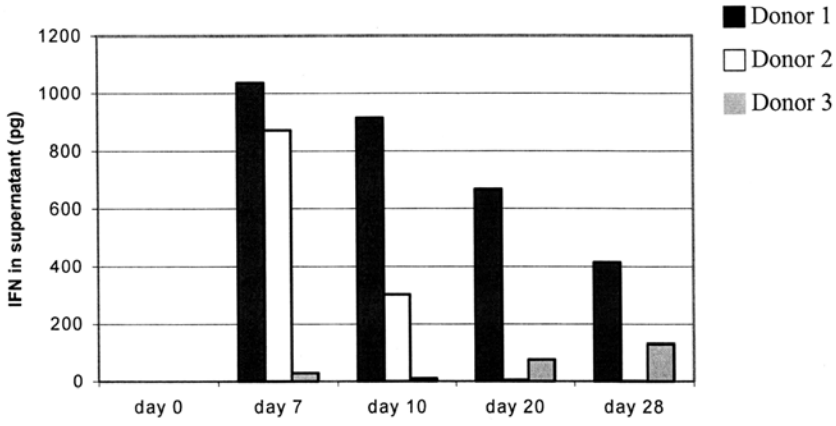

Figure 2. The levels of interferon-γ (IFN) in the supernatant of T cell cultures from 3 donors. In donor 3, low numbers of PBMC-derived dendritic cells were used for stimulations (see text).

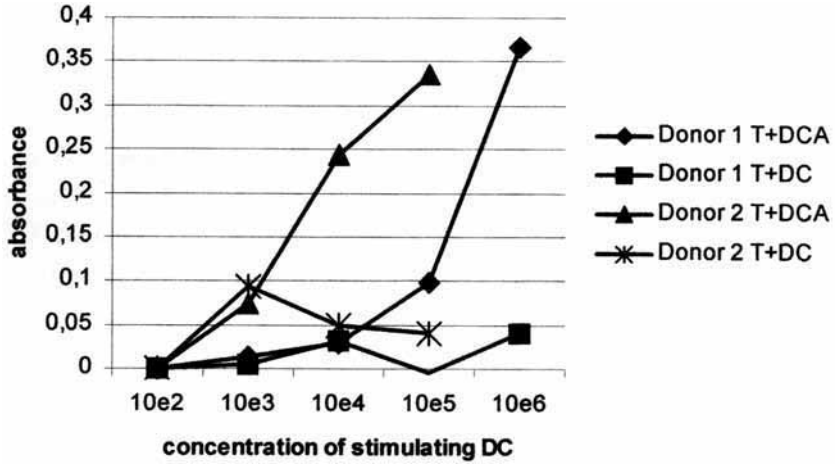

Figure 3. Proliferative response of restimulated T cells to antigen presented by autologous DCs – mean absorbances at 10 hours of culture of T cells from donors 1 and 2 with irradiated autologous DCs presenting the Id-KLH conjugate and with DCs not pulsed with the conjugate.

Proliferative response of restimulated lymphocytes to antigen presented by autologous DC and the cytotoxicity assay. On day 27, cells from lymphocyte cultures were used for a non-radioactive proliferation assay with Alamar Blue and a standard ^{51}Cr release cytotoxicity assay. The cells from donors 1 and 2 proliferated moderately in response to irradiated autologous antigen-loaded DCs but not in response to DCs that had not been pulsed with the antigen (Fig. 3). No proliferative response was seen in cells from donor 3 due to low numbers of DCs used for stimulations during culture (data not shown). In the cytotoxicity assays, the restimulated lymphocytes were added to ^{51}Cr-marked autologous DCs that were loaded with the Id-KLH conjugate. No cell lysis was seen – a further evidence of insufficient activity of CD8+ T cells generated during the restimulations.

Discussion

The successful *ex vivo* generation and expansion of tumor-specific CTLs in an autologous setting would greatly facilitate the development of new immunotherapeutical protocols. However, there are numerous problems that will need to be addressed before the method is applicable in the clinic.

Our results show that although T cells can be generated in a long-term culture using dendritic cells for repeated stimulation, their activity and IFN-γ is low after 4 weeks of culture and expansion by IL-2. While some degree of proliferation after stimulation with autologous antigen-presenting cells was detected, no lysis could be demonstrated in the autologous setting.

In two of three *in vitro* lymphocyte cultures we have seen decreases in the ratio of CD8 to CD4 cell. Although significant increase in the supernatant levels of IL-12 was observed while IL-10 decreased in the supernatants from DC cultures, the question remains whether Th2 rather than Th1 responses were induced. Furthermore, regulatory CD4+CD25+ cells may have been formed during the culture that would suppress the CTL activity.

A cytotoxicity assay using tumor cells as targets would be the only direct *in vitro* proof of cytolytic activity of *ex vivo* cultured and expanded antitumor CTLs. DCs pulsed with tumor antigens have been used as surrogate target cells [22] but it is unclear how close is the correlation of autologous DC lysis and tumor cell lysis by cytotoxic effectors.

Numerous strategies were proposed that might prevent the development of anergic T-cell clones. The defects in costimulation may be repaired using viral vectors to introduce DNA encoding for CD80 and other costimulatory molecules into dendritic or tumor cells in an effort to stimulate specific T-cell proliferation, cytokine release, and induction of cytolytic activity against malignant cells [23]. Cytokines known to enhance cell-mediated immune responses such as GM-CSF or interleukin-2 may be administered systemically as adjuvants with the vaccines or conjugated to the antigen aiming to create an environment where specific immune responses are readily induced [11]. Immunostimulatory DNA or Flt-3 (FMS-like tyrosine kinase 3) have been used recently to modulate the antigen-presenting capability of

DCs *in vivo* [12]. Stronger tumor-rejection antigens are searched that would not only be tumor-specific but that could also be efficiently targeted to destroy tumor cells, leading to clinically significant tumor regression [18].

Immune system dysfunctions in cancer patients pose formidable obstacles for the use of protocols using autologous antigen-presenting cells and CTLs as a modality in cancer treatment. This immunosuppression may be due to systemic chemotherapy, compromised nutrition status, cytokine network dysregulation, functional changes in T cells, B cells, NK/LAK cells, and immunosuppresive microenvironment in the proximity of the tumor [6, 19]. The stimulation of T-cells in the absence of costimulatory signals which are poorly expressed on tumor cells leads to unresponsiveness and a state of antigen-specific tolerance [6, 18, 19].

In our previous experiments, we found significant differences in numbers of DCs that can be generated *in vitro* from healthy donors and/or in patients with multiple myeloma (data not shown). This interindividual variability further complicates the application of DCs for vaccination or CTL production and needs to be considered when designing clinical protocols.

In conclusion, despite some progress, *ex vivo* production of active, specific CTLs in numbers sufficient for cancer immunotherapy remains a challenge nad new, more efficient approaches are needed.

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