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ORIGINAL ARTICLE

Angela Märten · Tim Greten · Carsten Ziske
Sabine Renoth · Björn Schöttker · Peter Buttgereit
Frank Schakowski · Alexander von Rücker
Tilman Sauerbruch · Ingo G.H. Schmidt-Wolf

Generation of activated and antigen-specific T cells with cytotoxic activity after co-culture with dendritic cells

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Abstract Co-culturing of immunological effector cells with antigen-pulsed DC leads to an increase of cytotoxic activity against antigen-expressing tumour cells. Using this approach, we could detect up to 2.8% antigen-specific CTLs after co-culture with antigen-pulsed DC. However, the required high effector cell numbers remain a major obstacle in immunotherapy. In this study, we show an approach for generating activated and antigen-specific effector cells that enables us to decrease effector to target cell ratios. We used an interferon-γ secretion assay to enrich activated effector cells after co-culture with antigen-pulsed dendritic cells (DC). Purified immunological effector cells lysed 58.3% of antigen-expressing tumour cells at an effector to target ratio of 1:1. Furthermore, using MHC-IgG complexes, we enriched effector cells expressing antigen-specific T-cell receptor after co-culture with DC. Performing ELISpot, flow cytometry and TCR analysis, we could show a significant increase of activated and specific TCR-expressing effector cells after co-culture with DC.

Keywords Interferon type II secretion · Dendritic cells · MHC-IgG molecules · T cells · CEA

A. Märten · C. Ziske · S. Renoth · B. Schöttker · P. Buttgereit
F. Schakowski · T. Sauerbruch · I.G.H. Schmidt-Wolf (✉)
Department of Internal Medicine I,
Rheinische Friedrich-Wilhelms-University, Bonn, Germany

T. Greten
Department of Gastroneterology and Hepatology,
Medical University, Hannover, Germany

A. von Rücker
Department of Clinical Biochemistry,
Rheinische Friedrich-Wilhelms-University,
Bonn, Germany

I.G.H. Schmidt-Wolf
Medizinische Klinik und Poliklinik I,
Sigmund-Freud-Str. 25, 53105 Bonn, Germany

Introduction

We, as well as others [3, 13], have observed a relative resistance of solid tumour cells to immunological effector cells in vitro. This resistance may be one reason for the clinical phenomenon that these tumours withstand immunotherapeutic approaches in humans.

Dendritic cells (DC) could be used to overcome such a resistance. DC play a major role in the immune response to tumour-associated antigens (TAA). DC in the periphery capture and process antigens, express lymphocyte co-stimulatory molecules, migrate to lymphoid organs and secrete mediators to initiate immune responses [2]. DC can now be readily obtained in sufficient quantities to allow biological studies and clinical trials [16]. DC stimulate immunological effector cells, such as T lymphocytes against TAA.

We developed a protocol generating large numbers of efficient cytotoxic effector cells [20, 21, 22]. This cell population includes up to 20% natural killer (NK)-like T lymphocytes, besides cytotoxic T cells. On the basis of this mixture they are able to recognize and kill tumour cells via the adoptive and the innate mechanisms of the immune system. Immunological effector cells were generated by incubation of peripheral blood lymphocytes with anti-CD3 monoclonal antibody, interleukin-2, interleukin-1β and IFN-γ. These effector cells possess an enhanced cytotoxicity and a higher proliferation rate compared with LAK cells [7, 17, 18].

Secretion of IFN-γ is a valid marker for activated T cells. We used IFN-γ secretion assays and IFN-γ ELISpots for determination of the percentage of activated T cells after co-culture with DC. Activated cells can be enriched using MACS technique.

Pulsing with immunogenic peptide or protein of human antigens allows us to generate activated, antigen-specific T cells. However, the vast majority of these peptide-specific CTL clones are peptide-reactive, but not tumour-reactive [11]. Recently, a peptide MHC ligand for a given population of T cells could be multimerised to get soluble peptide MHC tetramers and dimers [1, 8]. The percentage of antigen-specific T cells can be analysed using MHC-IgG molecules [1]. This dimer consists of an immunoglobulin of the IgG1 subtype and the C-terminus of an HLA-A2 molecule loaded with a specific peptide. When the peptide binds to HLA-A2, a complex is formed that is recognised specifically by the TCR of an antigen-detecting T cell. The immunoglobulin part of the dimer can be detected with a marked antibody. The T cells involved can be further characterised by using additional antibodies. We are therefore able to monitor and sort antigen-specific CTL in vivo and in vitro [4, 5, 8, 9, 11, 23, 24].

Here, we tested the effect of pulsed DC on immunological effector cells with respect to the generation of specific TCR, using dimeric MHC-IgG molecules for detection. It was found that co-culture of DC with immunological effector cells led to a significant increase of peptide-specific T cells. In addition, co-culture of pulsed DC and immunological effector cells led to a significant increase in cytotoxic activity of immunological effector cells against tumour cells.

Materials and methods

Generation of dendritic cells

Peripheral blood mononuclear cells were isolated from HLA-A2-positive buffy coats or from healthy donors by Ficoll density gradient centrifugation. Blood was drawn according to our protocol, which was accepted by the local ethics committee. The cells were allowed to adhere in six-well plates at a density of 5×10^6 cells/ml for 1 h at 37 °C in RPMI-1640 with 10% autologous, heat-inactivated serum. Non-adherent cells were collected for generating immunological effector cells (see below). The adherent cells were cultured in 2 ml RPMI-1640 with autologous, heat-inactivated serum, 750 U/ml human GM-CSF and 500 U/ml human IL-4 per well for seven days.

Pulsing of dendritic cells with protein or peptide

Dendritic cells were either pulsed on day +1 with 100 ng/ml CEA protein (Calbiochem, Bad Soden Germany) for three days, or with 1 µg CAP-1 (YLSGANLNL, immunological grade, Eurogentec, Hamburg, Germany) for 3 h. Pulsing was stopped by change of medium.

Generation of immunological effector cells and co-culture with DC

Immunological effector cells were generated as described previously [20, 22]. Briefly, non-adherent, Ficoll-separated human peripheral blood mononuclear cells were prepared and grown in RPMI-1640 medium, consisting of 10% fetal calf serum, 25 mM hydroxyethylpiperazine ethane sulphonic acid, 100 U/ml penicillin and 100 µg/ml streptomycin. Human recombinant interferon-γ was added on day 0. After 24-h incubation, 50 ng/ml of an antibody against CD3 (Orthoclone OKT 3, Cilag, Sulzbach, Germany), 100 U/ml IL-1β and 300 U/ml IL-2 (Boehringer) were added. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2, every 3 days 300 U/ml IL-2 were added.

Immunological effector cells were harvested on day +7 and were co-cultured for 7 days with autologous dendritic cells using a stimulator to responder ratio of 1:5. This concentration had been titrated and found to be optimal. Past the co-culture, effector cells and DC were grown in RPMI-1640 consisting of 10% fetal calf serum and supplemented with 300 U/ml IL-2 every third day.

After MACS-enrichment, effector cells were grown in RPMI-1640 consisting of 10% fetal calf serum and were supplemented with 3,000 U/ml IL-2.

Cell lines

Colo 205 (a colon carcinoma cell line) was purchased from European collection of cell cultures. The cells express CEA (CD66e) on their surface. The cell line is HLA-A2-positive. The cells were maintained in RPMI-1640 supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin.

Flow cytometry

Cells were incubated with the corresponding antibodies for 15 min on ice. The cells were then washed with phosphate-buffered saline containing 1% BSA. Dual-colour flow cytometric analysis was performed on a Coulter Epics XL cytometer.

Data were collected from 30,000 cells and analysed. DC were phenotyped with the following monoclonal markers: CD1a, CD80, CD86, HLA-ABC, HLA-DR (all from Pharmingen, Hamburg, Germany), CD83 and CMRF-44 (a kind gift from DNJ Hart, New Zealand). Negative controls consisted of DC labelled with mouse IgG. Immunological effector cells were phenotyped with antibodies against CD3, CD4, CD8, CD16, CD56, HLA-ABC and HLA-DR. CEA expression was determined by antibody against CD66e.

Analysis of IFN-γ-producing immunological effector cells using MACS cytokine secretion assay

IFN-γ-secreting cells were detected and enriched using the MACS cytokine secretion assay (Milteny, Berg. Gladbach, Germany) according to the manufacturer's instructions. In brief, IFN-γ catch reagent was attached to the cell surface via CD45 antibody. Secreted IFN-γ binds to the catch reagent during a 45-min incubation period. IFN-γ-specific detection antibody conjugated to PE was added, and anti-PE microbeads were used for enrichment on Vario MACS columns.

Analysis of IFN-γ producing immunological effector cells using ELISpot assay

The ELISpot technique is used to detect cytokine production by single cells [25]. In particular, IFN-γ production of immunological effector cells was determined by using a human IFN-γ ELISpot kit. Immunological effector cells were plated on nitrocellulose 96-well plates coated with an anti-IFN-γ antibody, at a concentration of $1\text{–}5 \times 10^4$ cells. Cells were incubated with 20 µg/ml of phytohemagglutinin and incubated for 48 h. After removal of cells, bound IFN-γ could be detected via a secondary biotinylated antibody. Streptavidin alkaline phosphatase binds to biotin and is detected via BCIP/NBT substrate. Native immunological effector cells, immunological effector cells co-cultured with pulsed and unpulsed DC, and immunological effector cells co-cultured with DC and tumour cells were assayed. Tumour cells were added at an effector to target cell ratio of 20:1 to co-cultured effector cells 24 h before starting the ELISpot assay. No Colo 205 tumour cells could be detected after 24 h. Blue spots were counted with a binocular microscope. No spots could be detected in the negative control.

Dimer technique

With the dimer technique, T cells with a specific receptor can be detected by flow cytometry. The amino acid sequence of the epitope and the HLA type must be known. Receptor-specific cells can be further characterised by additional staining [8]. Here, a chimeric protein, consisting of an immunoglobulin part and a HLA-A2 receptor part, was loaded with an HLA-A2-specific peptide (sequence YLSGANLNL). Ten microlitres of dimer were loaded with 3.2 μg peptide for 14 days at 4 °C. The peptide was synthesised by Eurogentec (Hamburg, Germany) and suspended in PBS with 0.01% sodium azide. The staining for CD8 and the specific TCR was performed similarly to the protocol described above. Cells (10^7) were incubated with 10 μl peptide-loaded dimer for 40 min on ice. Ten microlitres of anti-mouse IgG1-PE was added after washing and incubated for 20 min on ice to detect the immunoglobulin part of the dimer. After washing, 10 μl anti-CD8-FITC (IgG_2) was added and incubated for 20 min at 4 °C. The pellet was resuspended in 500 μl PBS with 1% BSA. At least 10^6 cells were analysed. A dimer loaded with an irrelevant HLA-A2-binding peptide (PepCD19 with the sequence IFAAQEL) was used as a control. The gates of the flow cytometer were set approximately to zero by using the irrelevant peptide. Anti PE-microbeads were used for MACS enrichment of CAP-1 specific-TCR expressing cells.

Cytotoxicity assay

A CytoTox 96 non-radioactive assay was used to measure cytotoxic activity. This assay is a colorimetric alternative to the ^{51}Cr-release assay. This quantitatively measures lactate dehydrogenase (LDH), which is released upon cell lysis in the same way as ^{51}Cr is released. Released LDH in culture supernatants was measured in a 30-min incubation using a coupled enzymatic assay. The density of the colour formed is proportional to the number of lysed cells. Absorbance data were collected using a 96-well plate reader set at 490 nm. Target cells (20,000) were plated in triplicates in a U-bottom 96-well tissue culture plate and incubated for 4 h with various ratios of effector to target cells. Fifty-microlitre aliquots from all wells were transferred to a fresh 96-well plate after incubation. Fifty microlitres of the substrate mix was added to each well of the plate and incubated at room temperature for 30 min in the dark. Fifty microlitres of a stop solution was added to each well before measuring. Target cells were Colo 205 cells. Maximal release of LDH was performed by incubating the target cells with 0.1% Igepal (anionic detergents from Sigma, Deisenhofen, Germany). Target cells without effector cells were used as negative control (spontaneous release). The cytotoxicity was calculated using the following formula:

$$\begin{aligned}&\text{percentage cytotoxicity}\\&\quad=[(\text{experimental absorbance}-\text{spontaneous release of effector cells})\\&\qquad-\text{spontaneous release of target cells}]/(\text{maximal release}\\&\qquad-\text{spontaneous release of target cells})]\times 100.\end{aligned}$$

Statistical analysis

Unpaired *t*-test was used to analyse the statistical significance. A P value <0.05 was considered significant.

Results

In vitro generation of DC

DC were generated from buffy coats using GM-CSF and IL-4 as described in Materials and methods. After 7 days of culture, the yield of DC generated was 25% of the starting PBMC population. Adherent cells showed cytoplasmic processes as described for DC. They expressed 35.0 ± 7.0% CD83, 50.9 ± 2.9% HLA-DR and 85.4 ± 2.9% HLA-ABC on day +13 and were negative for CD14 expression, as determined by flow cytometry. After co-culturing with immunological effector cells, typical non-adherent clusters were formed.

In vitro generation and immunophenotyping of immunological effector cells

Immunological effector cells were generated as described before [22]. The phenotypic analysis results were similar to previous reports [20, 21]. We found an enhanced proliferation of immunological effector cells after contact with DC. Between days +7 and +14 the number of effector cells doubled when co-cultured with DC (an increase in proliferation rate of 2.03 ± 0.17 times, $n=11$). The absolute cell number increased by a factor of 2.84 ± 0.4% from day 0 to day +14. Furthermore, during culture effector cells showed an increase in percentage of the cytotoxic T cells. At day +8 of culture, the $CD8^+$ $CD3^+$/$CD4^+$ $CD3^+$ ratio of non-co-cultured effector cells was 0.77 ± 0.12; at day +14 the ratio was 1.06 ± 0.1. Immunological effector cells that had been co-cultured with dendritic cells at day +7 showed a highly significant increase of the ratio ($P=0.0001$). At day +8, the ratio was 0.98 ± 0.01, and at day +14 it was 1.52 ± 0.06 (Fig. 1).

Analysis of IFN-γ producing immunological effector cell cultures by MACS cytokine secretion assay

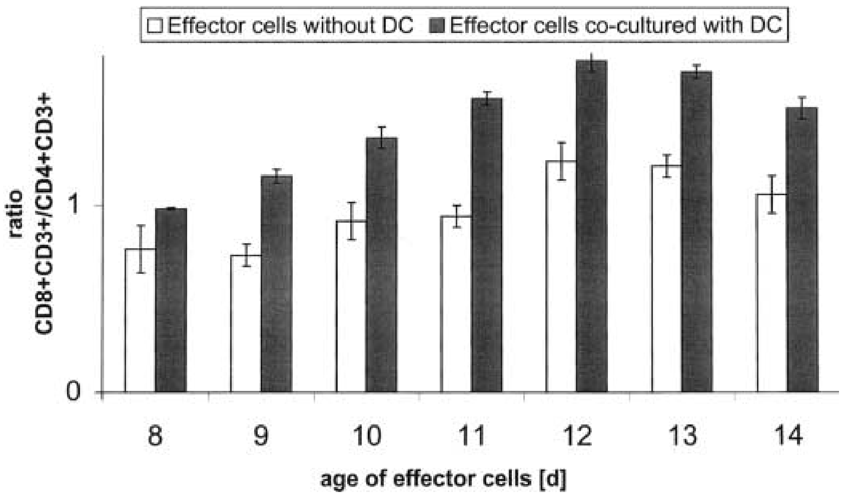
IFN-γ-producing effector cells were also determined by MACS cytokine secretion assay (Fig. 2A, B). In native effector cell populations 0.8 ± 0.4% IFN-γ-producing cells could be detected (Fig. 2A). Co-culture of immunological effector cells with unpulsed DC led to an increase of IFN-γ-secreting effector cells (0.95 ± 0.25%). After co-culture with CEA-pulsed DC the percentage of IFN-γ-producing immunological effector cells further increased and was 6.2 ± 1.0% ($P<0.05$).

After MACS enrichment, 67.4 ± 21.7% of the cells were positive for IFN-γ secretion (Fig. 2B). The enriched population was double-positive for IFN-γ secretion and CD8 expression at a level of 34.9 ± 1.7%. The percentage of CD8 cytotoxic cells was 38.2 ± 5.8%.

Analysis of IFN-γ producing immunological effector cell cultures by ELISpot

IFN-γ production by immunological effector cell cultures was determined by the ELISpot technique. Only a few IFN-γ producing cells could be detected in native effector cell cultures (3.0 ± 0.2 of 10^4 cells; Table 1). Coculture of immunological effector cell cultures with DC led to a significant increase of IFN-γ secreting cells (45 ± 12 of 10^4 cells with unpulsed DC, $P<0.001$; 57 ± 10 of 10^4 cells

Fig. 1 Ratios of $CD8^+$ $CD3^+$ to $CD4^+$ $CD3^+$ cells over time. Effector cells were either co-cultured or not co-cultured from days +7 to +14 with autologous DC. The percentages of $CD8^+$ $CD3^+$ and $CD4^+$ $CD3^+$ lymphocytes were determined by flow cytometry at the indicated time-points. Data represent results from three separate experiments and are shown as mean ± SE



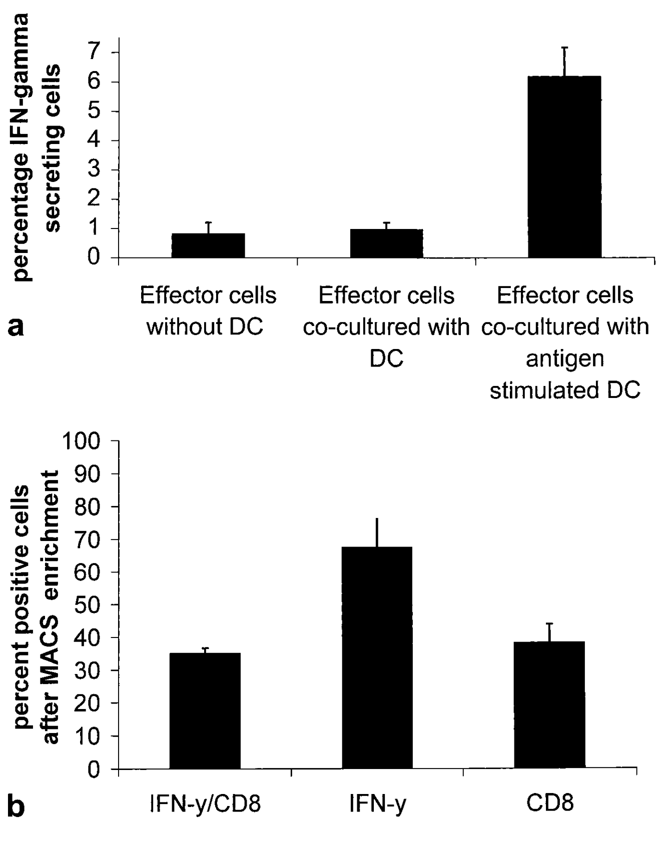


Fig. 2A, B Purity of immunological effector cells after enrichment of IFN-γ secreting cells. **A** The percentage of activated cells in the whole effector population before MACS enrichment. DC were either unstimulated or pulsed with 100 ng/ml CEA. **B** Immunological effector cells were enriched on day +14 by using the IFN-γ secretion assay after co-culture from days +7 to +14 with autologous DC. After enrichment, the effector cells were stained with monoclonal antibody directed against CD8. Data represent results from three separate experiments and are shown as mean ± SE

with antigen-pulsed DC, $P=0.07$), co-culture with antigen-expressing tumour cells (Colo 205) led to a further increase to 128 ± 12 of 10^4 cells (Table 1).

Analysis of T cell receptor specificity

We investigated whether co-culturing of immunological effector cells with specifically pulsed DC led to an increase in the number of antigen-specific T cells. As a control, the dimer was loaded with an irrelevant peptide in order to determine unspecific binding. PepCD19, which binds to HLA-A2, was used as an irrelevant peptide, since no interaction of this B cell marker with T-cell receptor (TCR) was expected. TCR specificity of effector cell cultures was analysed before and after co-culture with DC pulsed with CAP-1 peptide. T cells with specific TCR were determined by flow cytometric analysis. As depicted in Fig. 3, cytotoxic T cells expressing CD8 were gated.

As a control, a dimer with an irrelevant PepCD19 peptide was added to immunological effector cells co-cultured with CAP-1 peptide-pulsed DC. The percentage of the cells that co-expressed CD8 and PepCD19 was 0.29 ± 0.04% (Fig. 3A). In contrast, 1.15 ± 0.2% of immunological effector cells co-cultured with CEA-pulsed DC co-expressed CD8 and CAP-1 specific TCR (Fig. 3B3; $P=0.08$). Interestingly, with CAP-1-pulsed DC, this percentage increased to 1.62 ± 0.2% (Fig. 3C; Table 2; $P=0.049$).

The percentage of receptor-specific lymphocytes was also determined independently from the CD8 expression, as not only cytotoxic T cells express receptors for antigen detection. Similarly, the PepCD19 control showed an expression of 0.55 ± 0.45% specific TCR. After co-culture with CEA pulsed DC, the percentage increased to 1.45 ± 0.02% ($P=0.24$); after co-culture with CAP-1 peptide-pulsed DC it rose to 2.00 ± 0.42% ($P=0.39$; Table 2).

The percentage of antigen-specific cytotoxic cells of the $CD8^+$ subset was of particular interest. Here, unspecific dimer led to 0.9 ± 0.0%, CEA pulsed DC led to 2.76 ± 0.01% and CAP-1-pulsed DC led to 3.72 ± 0.15% (Table 2).

Table 1 ELISpot analysis of IFN-γ-producing immunological effector cells. Immunological effector cells were generated as described and co-cultured for 7 days with DC. DC were pulsed with an irrelevant protein (myoglobin) or CEA protein. Colo 205 cells, which are CEA-positive, were added one day before performing ELISpot. Effector cells without co-culture with DC are shown as control. Data are shown as mean ± SE from two separate experiments

	Spots per 10^4 cells
Effector cells	3 ± 0.2
Effector cells co-cultured with DC	45 ± 12
Effector cells co-cultured with DC pulsed with myoglobin	47 ± 10
Effector cells co-cultured with DC pulsed with CEA protein	57 ± 10
Effector cells co-cultured with DC and Colo 205 cells	128 ± 12

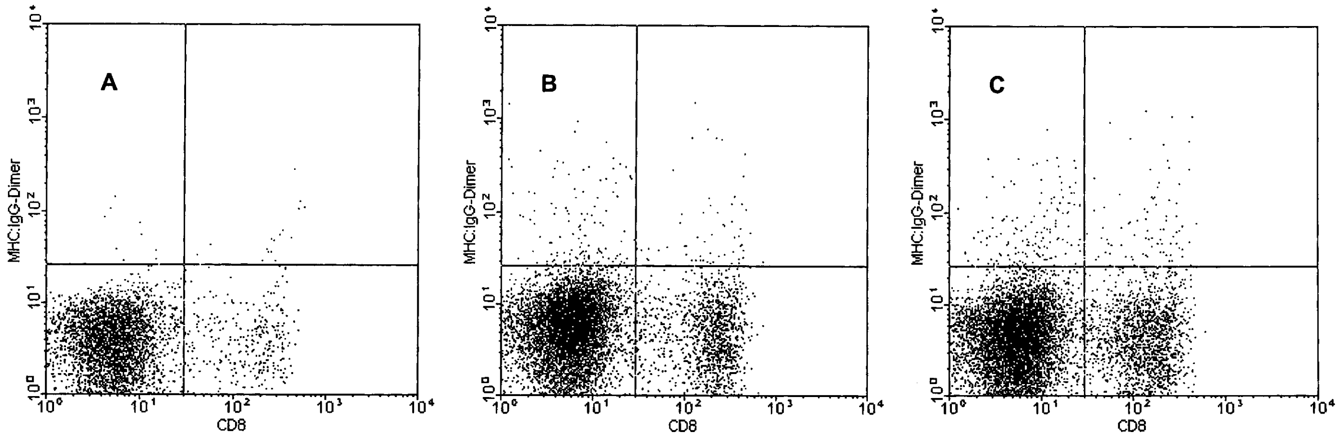


Fig. 3A–C Flow cytometric analysis of CAP-1 specific immunological effector cells after co-culture with pulsed DC. CD8$^+$ cells are shown on the *x-axis* and bound dimer detected by PE-staining against IgG1 on the *y-axis*. **A** As a control, immunological effector cells were co-cultured with DC pulsed with CAP-1, and dimer loaded with the irrelevant peptide was detected. Flow cytometric analysis of CAP-1 specific TCRs on immunological effector cells after co-culture with DC pulsed with CEA protein (**B**) or CAP-1 peptide (**C**) are shown

Stimulatory activity of DC on cytotoxic immunological effector cells activity

We investigated whether the increase in activated and antigen-specific effector cells after co-culture with dendritic cells leads to increased lytic activity. Immunological effector cells were co-cultured from days +7 to +14 with DC cultures 7 days of age and cytotoxicity of effector cells was analysed. Co-culture of effector cells with DC led to a significant increase in cytotoxic activity as measured in a LDH release assay using Colo 205, a colon carcinoma cell line, as target. For example, LDH release in co-cultures was 46.0 ± 8.0%, compared with 0% using effector cells alone at an effector to target cell ratio of 25:1 (Fig. 4; $P = 0.037$). Pulsing of dendritic cells with tumour-associated protein (CEA) on day +1 for 72 h led to a further increase, the measured cytotoxicity was 69.2 ± 14.0%. Similarly, dendritic cells were pulsed with CAP-1, an immunogenic epitope of CEA, on day +7 for 4 h. Co-culture of immunological effector cells with these DC led to a comparable cytotoxic activity. LDH release in co-cultures was 67.9 ± 8.3% at the same effector to target ratio as above. In contrast, effector cells without co-culture showed no lytic activity.

Cytotoxic activity of IFN-γ secreting immunological effector cells after MACS enrichment

Immunological effector cells were co-cultured from days +7 to +14 with DC cultures 7 days of age and activated cells were enriched using IFN-γ secretion MACS technique at day +14. Cells were cultured for expansion of activated cells for another six days with 3,000 U/ml IL-2, and the cytotoxicity of effector cells was analysed. Enriched cells showed moderate proliferation (factor 1.5–2.5) and increased cytotoxic activity compared to LDH-release assays using the whole population.

After enrichment, co-cultured immunological effector cells were able to lyse 26.4 ± 14.9% of Colo 205 cells at an effector to target ratio of 1:1. When using CEA-pulsed DC, co-cultured effector cells lysed 58.3 ± 9.2% of target cells ($P = 0.04$). However, effector cells without enrichment showed lytic activity lower than 5% (Fig. 5).

Cytotoxic activity of MACS-enriched CAP-1 specific TCR expressing effector cells

Immunological effector cells were co-cultured from days +7 to +14 with DC cultures 7 days of age and CAP-1-specific TCR expressing cells were enriched using MACS

Table 2 Flow cytometric analysis of peptide-specific TCR on $CD8^+$ cells. Dimer was loaded with CAP-1 peptide or as control with the irrelevant peptide pepCD19 over 14 days. DC were pulsed either with CEA protein on day +1 or CAP-1 peptide on day +5. Immunological effector cells were co-cultured with DC on day +7 for seven days. Values written in *bold* show the difference between dimer loaded with CAP-1 and the control. Data represent results from three separate experiments and are shown as mean ± SE

	$CD8^+$/peptide-specific TCR^+ (percentage from the whole population)	Peptide-specific TCR^+ (percentage from the whole population)	$CD8^+$/peptide-specific TCR^+ (percentage from $CD8^+$ cells)
Dimer loaded with pepCD19,	0.29 ± 0.04%	0.55 ± 0.17%	0.90 ± 0.0%
DC pulsed with CAP-1 (control)	**0.0%**	**0.0%**	**0.0%**
Dimer loaded with CAP-1,	1.15 ± 0.2%	1.45 ± 0.02%	2.76 ± 0.01%
DC pulsed with CEA protein	**0.86%**	**0.90%**	**1.86%**
Dimer loaded with CAP-1,	1.62 ± 0.2%	2.00 ± 0.42%	3.72 ± 0.15%
DC pulsed with CAP-1	**1.33%**	**1.45%**	**2.82%**

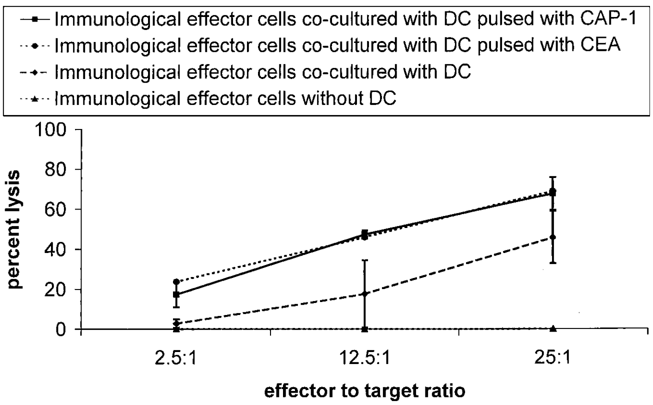


Fig. 4 Cytotoxic activity of immunological effector cells that had been co-cultured with DC against colon carcinoma cells. Immunological effector cells from an HLA-A2-positive donor were co-cultured from days +7 to +14 with autologous DC cultures seven days of age, as described in Materials and methods. DC were pulsed at day +1 with 100 ng/ml CEA over 72 h or at day +7 with 1 μg CAP-1 over 4 h. Cytotoxic activity at various effector to target cell ratios was measured by LDH-release assay. Colo 205 cells were used as targets. Data represent results from three separate experiments and are shown as mean ± SE

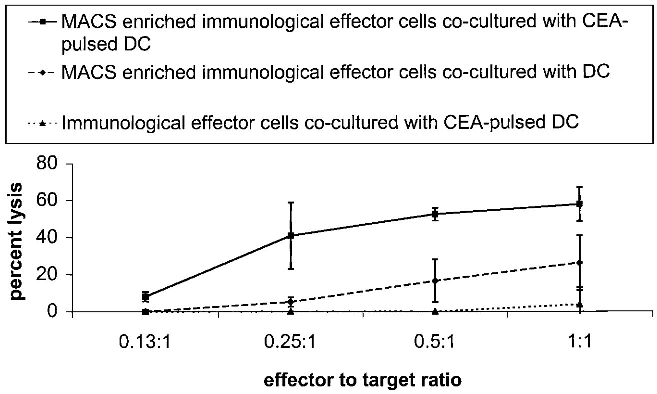


Fig. 5 Cytotoxic activity of immunological effector cells after enrichment of IFN-γ-secreting cells. Immunological effector cells were enriched on day +14 by using the IFN-γ secretion assay after co-culture with autologous DC from days +7 to +14. After enrichment, the effector cells were cultured for six further days as previously described, using the 10-fold concentration of interleukin-2. Cytotoxic activity at various effector to target cell ratios was measured by LDH-release assay. Colo 205 cells were used as targets. Data represent results from three separate experiments and are shown as mean ± SE

technique at day +14. Cells were cultured for expansion for another six days with 3,000 U/ml IL-2, and the cytotoxicity of effector cells was analysed. Enriched cells showed moderate proliferation (factor 1.5–2.5) and increased cytotoxic activity compared with LDH-release assays using the whole population.

After enrichment, immunological effector cells co-cultured with CAP-1-pulsed DC were able to lyse 80.1 ± 5.2% of Colo 205 cells at an effector to target ratio of 5:1. When using CEA-pulsed DC, co-cultured effector cells lysed 82.3 ± 22.3% of target cells (Fig. 6).

Discussion

We previously reported a protocol for generating large numbers of efficient immunological cells by incubation of peripheral blood lymphocytes with different cytokines [20, 22]. The resulting heterogeneous cell population consists of T, NK, and NKT cells [15], which are derived from T cells [10]. These effector cells are capable of promptly producing several cytokines, including IFN-γ [12].

Here, these effector cells were co-cultured with autologous dendritic cells. By using ELISpot and flow cytometry analysis, interactions between effector and dendritic cells, resulting in activation of effector cells, could be detected. Generally, a significant increase of activated effector cells after co-culture with DC was observed. One reason for this phenomenon could be the stimulation of effector cells with cytokines secreted by DC and interactions between co-stimulatory and antigen-presenting molecules on DC with corresponding molecules on effector cells. DC are able to activate T cells via IL-12 secretion, presentation of antigens in the context with MHC class I and II molecules, expression of B7 molecules, and CD40 and CD40L interactions. Activation of NK and NK-like T cells is based on IL-12 secretion and interaction between CD1 molecules and $V_{\alpha}24^+$ TCR on NK-like T cells [6, 14]. Using flow

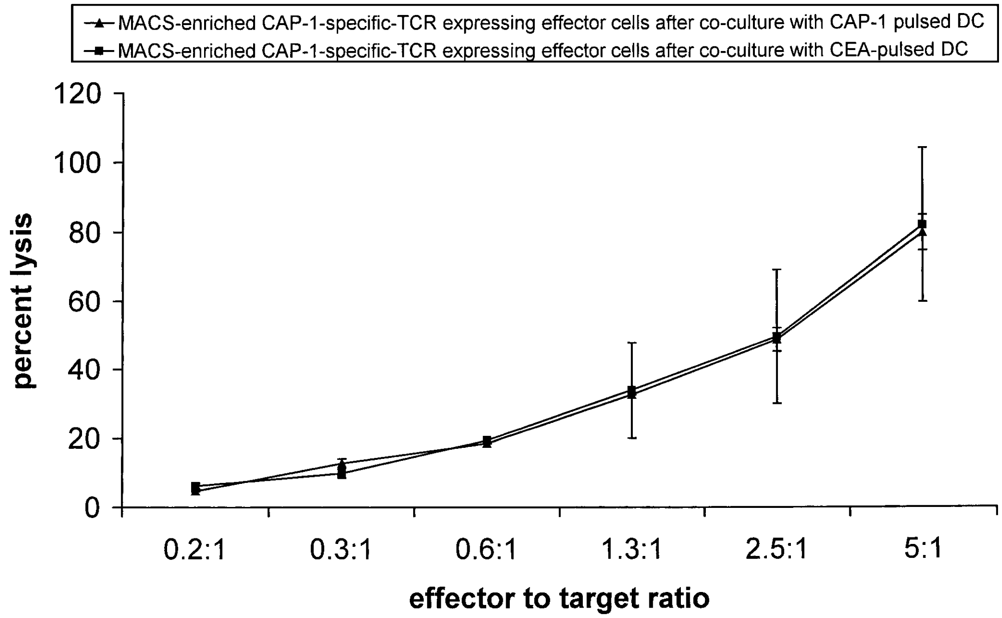


Fig. 6 Cytotoxic activity of immunological effector cells after enrichment of CAP-1-specific TCR-expressing cells. Immunological effector cells were enriched on day +14 by using MACS technique after co-culture from days +7 to +14 with autologous DC. After enrichment, the effector cells were cultured for six further days as previously described, using the 10-fold concentration of interleukin-2. Cytotoxic activity at various effector to target cell ratios was measured by LDH-release assay. Colo 205 cells were used as targets. Data represent results from three separate experiments and are shown as mean ± SE

cytometry, we observed an enhanced percentage of IFN-γ-positive cells compared with ELISpot results.

Recently, a peptide-MHC ligand for a given population of T cells could be multimerised to get soluble peptide-MHC tetramers and dimers [1, 8]. In general, tetramer-binding analysis correlated well with cytotoxicity assays [1]. Normal frequency of antigen-specific cells in human blood is between 10^{-4} and 10^{-5}. We could demonstrate that co-culturing of effector cells with antigen-pulsed DC led to an increase of antigen-specific T cells up to 3.7% (see Table 2). Pulsing DC with the defined epitope CAP-1 resulted in a higher increase of CAP-1-specific effector cells compared with the data from pulsing with CEA protein. This indicates that the different epitopes on CEA were resulting in an oligoclonal expansion of antigen-specific effector cells compared with a monoclonal expansion of CAP-1-specific cells after pulsing with CAP-1. Compared with our data, Lee et al. [11] described one patient with malignant melanoma as having 2.2% tyrosinase-specific T cells; but these cells were, contrary to our effector cells, anergic.

Comparing the data from MHC-IgG-complex measurements with the determinations of IFN-γ secreting cells after co-culture with CEA-pulsed DC, we observed more IFN-γ secreting cells than effector cells with TCR specific for CAP-1. This is not surprising, since CEA includes much more immunogenic epitopes than CAP-1, so that effector cells with different TCRs are activated by pulsing with CEA, resulting in IFN-γ secretion.

We showed recently that DC pulsed with tumour antigens like CEA are able to increase the cytotoxic effect of co-cultured immunological effector cells against colon and pancreatic tumour cell lines significantly and also against primary tumour cells derived from patients with metastatic colorectal cancer [11, 13]. We could show that an increase in activated, peptide-specific T cells correlates well with a significant increase in cytotoxicity against tumour cells.

Performing cytotoxicity assays after MACS enrichment of either activated or antigen-specific cells, we could decrease the required effector to target ratio for lysing tumour cells to a very low value. Enriched effector cells expressing CAP-1-specific TCR after co-culture with either CEA- or CAP-1-pulsed DC were equally cytotoxic. This supports our model, that pulsing with CEA protein resulted in a variety of antigen-specific effector cells, but only CAP-1-specific cells were enriched. After enrichment, activated effector cells showed a higher lytic capacity compared with antigen-specific cells. Presumably, these phenomena could be caused by antigen-specific effector cells in an anergic status, and enrichment of activated cells also involves TCR-negative cells (for example, NK-like T cells).

After enrichment of activated or antigen-specific cells we could induce a moderate propagation of these cells compared to the proliferation rate in the unpurified population. This allows not only a purification of the cells of interest, but enabled us to generate the required cell numbers.

In view of clinical application, we should mention that immunological effector cells can be generated after leukapheresis in large quantities of up to 10^9 cells [19]. Generation of 3% peptide-specific T cells correlates with 3×10^7 peptide-specific and tumour-lysing T cells obtainable by a single apheresis. Further experiments will be performed to verify this approach.

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