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Enhanced lytic activity of cytokine-induced killer cells against multiple myeloma cells after co-culture with idiotype-pulsed dendritic cells

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Background and Objectives. There are numerous reports of *in vitro* and *in vivo* usage of dendritic cells (DC) pulsed with idiotype, the tumor-specific antigen of multiple myeloma (MM), for immunotherapy of MM. Data suggest that not only T-cells, but also the innate immune system reacts against MM. Here, we examined the cytotoxic activity of cytokine-induced killer (CIK) cells against myeloma cells. This heterogeneous effector population consists of T-, NK- and NKT-cells.

Design and Methods. CIK cells generated from buffy coats or blood from patients with MM were co-cultured with autologous idiotype-pulsed DC. The cytotoxic activity was investigated in lactate dehydrogenase release assays against cell lines or autologous CD138 positive cells from bone marrow.

Results. CIK cells were able to lyse MM cells at low effector to target ratios. This effect was significantly enhanced by co-culturing with specifically pulsed DC (83.8% lysis at an effector to target ratio of 16:1). Using an interferon-γ secreting MACS separation assay, the cytotoxic activity of CIK cells was enhanced to maximal lysis at the lower effector to target ratio of 5:1. High cytotoxic activity was also shown in a completely autologous setting against enriched $CD138^+$ cells from a patient with MM (54.4% lysis at an effector to target ratio of 6:1). Interestingly, there was no cytotoxic activity against the $CD138^-$ fraction of the bone-marrow.

Interpretation and Conclusions. Using a heterogeneous population of effector cells, we were able to activate the innate and the adoptive immune-system against myeloma cells. CIK cells showed high lytic activity against MM cells, which could be enhanced by co-culturing with antigen-specific pulsed DC.

Key words: CIK-cells, dendritic cells, idiotype, multiple myeloma, immunotherapy.

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Multiple myeloma (MM) is a malignant disease characterized by clonal proliferation of plasma cells in the bone marrow.[1] Despite considerable progress in the understanding of its biology and introduction of high-dose chemotherapy the disease has remained largely incurable. Standard dose chemotherapy regimens result in median survival rates of two to three years. Considerable progress in the treatment of MM has been made over the past few years since high-dose chemotherapy with autologous stem cell transplantation (auto-SCT) was introduced as first-line therapy.[2] However, molecular remissions are rare and virtually all patients seem to relapse.[3] The only cure for MM still appears to be allogeneic SCT. This treatment is hampered by its severe toxicity and can, therefore, only be applied to a very small number of patients.[4,5] The chance of cure by allo-SCT is probably mediated by a graft-versus-myeloma (GVM) effect. However, the treatment-related mortality is very high in the first year despite considerable efforts to improve the safety of transplant procedures.[6] Since chemotherapy is not expected to offer cure or long-term disease control without considerable toxicity including long-term secondary malignancies,[7] the development of new therapeutic strategies is required.

In particular, patients with MM who received high-dose chemotherapy may profit from immunotherapeutic approaches due to the intense reduction of tumor load achieved with this treatment modality. The monoclonal immunoglobulin secreted by myeloma cells may serve as a tumor-specific antigen because of the unique antigenic structure in its variable regions (idiotype) and could be used for a novel therapeutic strategy.[8,9] Meanwhile, there are numerous reports on *in vitro* and *in vivo* usage of dendritic cells (DC) pulsed with idiotype. DC are antigen-presenting cells of the immune system which prime T-cell reactivity towards malignant cells.[10] Several studies demonstrate the presence of

a T-cell mediated immune response against MM.[11,12] DC with normal phenotype and function can be generated from patients with myeloma.[13] With regard to data which also suggest that the innate immune system plays a role in MM,[14,15] we examined the cytotoxic activity of cytokine-induced killer (CIK) cells[16-18] co-cultured with idiotype-pulsed DC against MM. CIK cells consist of T-, NK- and NKT-cells. These NKT-cells are non-major histocompatibility complex-restricted cytotoxic lymphocytes with an enhanced cytotoxicity and a higher proliferation rate than LAK cells.[19,20]

Design and Methods

Purification of idiotype

Idiotype was precipitated over 30 min from serum or cell culture supernatant using 40% ammonium-sulphate (AS). The solution was centrifuged at 20,000×g for 10 min, followed by a dialysis repeated twice overnight at 4°C (10 mM potassium-phosphate, 50 mM NaCl, pH 7.0). The dialysate was purified on a protein-L sepharose column (Pierce, Rockford, USA) according to the manufacturer's instructions. The concentration of myeloma protein was determined spectrophotometrically at 280 nm using an extinction coefficient of 1.4.

Immunoblotting

For SDS-page analysis each lane was loaded with 10 μg of protein (1-8 μL) and electrophoretically separated on 10% polyacrylamide-SDS gel. Proteins were transferred to nitrocellulose by semi-dry blotting. Ponceau staining was performed to show the relative quantities of protein samples loaded. The membranes were blocked with 0.5% Western blocking reagent (Roche, Mannheim, Germany) in triphosphate buffered saline (TBS) and probed with a 1:10,000 dilution of peroxidase-conjugated affinipure goat anti-human IgG (Dianova, Hamburg, Germany) antibody. The membranes were developed according to the Pierce Supersignal protocol (Pierce).

Generation of DC

Peripheral blood lymphocytes were isolated from buffy coats from healthy donors or from blood from patients with MM, as indicated, by Ficoll density gradient centrifugation (Lymphoprep, Nycomed, Oslo, Norway). Blood was drawn according to our protocol 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 one hour at 37°C in RPMI 1640 with 10% autologous, heat-inactivated serum. The non-adherent cells were collected for generating CIK cells (see below). The adherent cells were cultured in 2 mL RPMI 1640 with autologous, heat-inactivated serum, 750 U/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) and 500 U/mL interleukin (IL)-4 (Essex Pharma, Nuremberg, Germany) per well.

Generation of CIK cells

CIK cells were generated as described previously.[16,19] In brief, non-adherent Ficoll separated human peripheral blood mononuclear cells were prepared and grown in RPMI 1640 medium (Gibco BRL, Karlsruhe, Germany), consisting of 10% fetal calf serum (PAA, Cölbe, Germany), 25 mM Hepes, 100 U/mL penicillin and 100 μg/mL streptomycin (Seromed, Berlin, Germany). On day 0, 1000 U/mL human recombinant interferon-γ (Roche, Mannheim, Germany) were added. After 24 hrs of incubation, 50 ng/mL of an antibody against CD3 (Orthoclone OKT 3, Cilag GmbH, Sulbach, Germany), 100 U/mL interleukin-1β and 300 U/mL interleukin-2 (Roche) were added. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and subcultured every three days in fresh complete medium and IL-2 at 3×10^6 cells/mL.

Pulsing of DC

DC were pulsed on day +1 with either serum or purified idiotype containing 100 μg/mL protein. As control they were pulsed with 100 μg/mL myoglobin (Sigma, Deisenhofen, Germany) as irrelevant protein. Pulsing was stopped on day +4 by changing medium.

Co-culturing of CIK cells with autologous DC

CIK cells were harvested on day +7 and were co-cultured for another seven days with autologous seven-day old DC at a stimulator to responder ratio of 1:5.

Enrichment of interferon (IFN)-γ producing immunologic effector cells using MACS cytokine secretion assay

IFN-γ secreting cells were detected and enriched using the MACS cytokine secretion assay (Miltenyi, Bergisch 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. The cytotoxic potential of enriched cells was investigated within 48hrs after enrichment.

Cytotoxicity assay

A CytoTox 96 non-radioactive assay (Roche) was used to measure cytotoxic activity. This assay is a colorimetric alternative to the ^{51}Cr release assay. It 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 color formed is proportional to the number of lysed cells. Absorbency data were collected using a 96-well plate reader set to 490 nm. Target cells (10,000) were plated in triplicate sets in a U-bottom 96-well tissue culture plate and incubated for 4hrs with various ratios of effector to target cells. After incubation 50 μL aliquots from all wells were transferred to a new 96-well plate. To each well of the plate, 50μL of the substrate mix was added and incubated at room temperature for 30 min in the dark. Before measuring, 50 μL of a stop solution was added to each well. Target cells were either the cell line OPM or for the complete autologous setting CD138 positive cells from bone marrow. Maximal release of LDH was achieved by incubating the target cells with 0.1% IGEPAL (anionic detergent from Sigma). The negative control (spontaneous release) was target cells without effector cells. The ratio between maximal and spontaneous release was >5.

Lytic units were defined as 10^7 effector cells divided by the number of effector cells which are required to cause 20% lysis of 10^4 target cell (as the formula below).[21]

$$\frac{10^7}{X_E \times T}$$

The cytotoxicity was calculated using the formula:

$$\text{cytotoxicity} = \frac{\text{Exp. LDH Rel - Sp. Rel EC - Sp. Rel TC}}{\text{maximal Rel. - Sp. Rel of TC}}$$

where: Exp. = experimental; Rel = release; Sp. = spontaneous; EC = effector cells; TC = target cells.

Isolation of myeloma cells from bone marrow

Myeloma cells from bone marrow aspirates were isolated from a patient with MM (idiotype IgG) by Ficoll density gradient centrifugation. After washing, the cells were enriched using CD138 microbeads (Miltenyi) according to the manufacturer's instructions. CD138 cells were tested within 24hrs after enrichment as targets in a LDH release assay.

Cell lines

MM cell lines OPM-2 (secreting IgG) and U266 (secreting IgE) were purchased from DSMZ (Deutsche Sammlung für Mikroorganismen und Zellkultur, Braunschweig, Germany). 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 on ice for 15 min and were then washed with PBS/1% BSA (PBS for phosphate-buffered saline, PAA; BSA for bovine serum albumin from Sigma). Dual-color flow cytometric analysis was performed on a Coulter Epics XL Cytometer (Coulter-Immunotech, Krefeld, Germany).

Data were collected from 30,000 cells and analyzed. DC were phenotyped with the following monoclonal markers: CD1a, CD80, CD86, HLA-ABC, HLA-DR (all from Pharmingen, Hamburg, Germany), CD83 (Coulter-Immunotech). Negative controls consisted of DC labeled with mouse Ig (PE/FITC). CIK cells were phenotyped with antibodies against CD3, CD8, CD28 and CD56.

Statistical analysis

Unpaired t-test were used to analyze for statistical significance. A p value < 0.05 was considered statistically significant.

Results

Purification of idiotype and immunoblotting

After purifying idiotype using AS-precipitation and protein sepharose L, we observed an increasing purity of immunoglobulin (Figure 1 a/b).

In vitro generation of CIK cells

CIK cells were generated as described in the Design and Methods section. Results of phenotypic analysis were similar to those in previous reports.[16,17] CIK cells expressed CD3 and the α/β T-cell receptor. A subpopulation co-expressed CD56, but was mainly negative for CD16. Apart from $CD4^+$ and $CD8^+$ cells, the population included 10 to 15% $CD3^+CD56^+$ cells on day +14 (Figure 2a). The percentage of these double-positive cells increased significantly during further cultivation. CIK cells were mainly positive for CD28 expression.

CD3, CD4, CD28 and CD40L expression of CIK cells increased after co-culture with DC. In contrast, there was no significant change of HLA-ABC and HLA-DR expression with and without co-culture of DC (HLA-ABC and HLA-DR expression was stable at 99.7±0.3% and 61.8±1.2%, respectively after 13

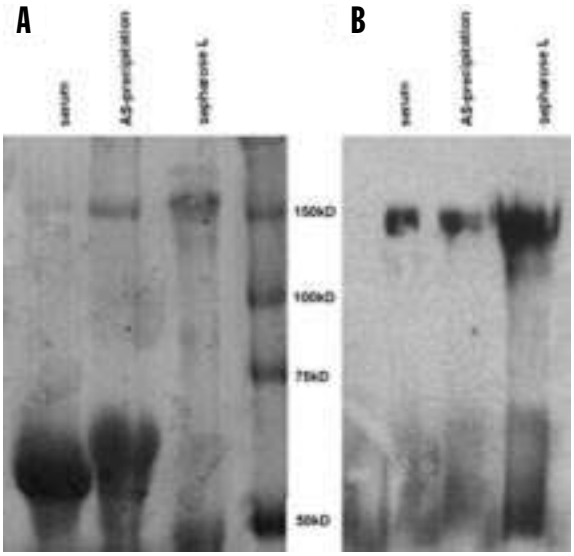


Figure 1. Immunoblotting of purified idiotype. Each lane was loaded with 10 µg protein. Protein was stained using Ponceau solution. Immunoglobulin was detected using peroxidase-conjugated anti-IgG antibody.

days of culture [data not shown]). Proliferation rate increased after contact with DC. Between day +7 and day +14 the number of CIK cells doubled when co-cultured with DC (increase in proliferation rate 2.03±0.17; n=11), this being mainly due to proliferation of $CD8^+$ cells (Figure 2b).

In vitro generation of DC

DC were generated from buffy coats using GM-CSF and IL-4 as described in the *Design and Methods* section. After 14 days of culture the yield of DC generated was 25% of the starting peripheral blood mononuclear cell population. Adherent cells showed cytoplasmic processes typical of DC. After co-culturing with CIK cells the cells formed typical clusters. Flow cytometry showed CD14 negative populations, expressing markers typical of DC (Figure 3).

Effect of pulsing DC with idiotype on cytotoxic activity of CIK lymphocytes

DC were pulsed with idiotype or with an irrelevant protein as control for three days and were used to stimulate CIK lymphocytes. Cytotoxicity was assessed using OPM-2 cells as targets. CIK cells showed low lytic activity (16±6 lytic units), which could be improved by co-culturing with DC. To induce specific cytotoxicity, DC were pulsed with protein derived from the target cell line. The highest cytotoxicity was detected by pulsing with supernatant purified by using AS precipitation and

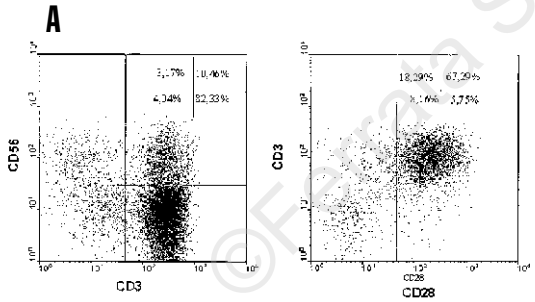
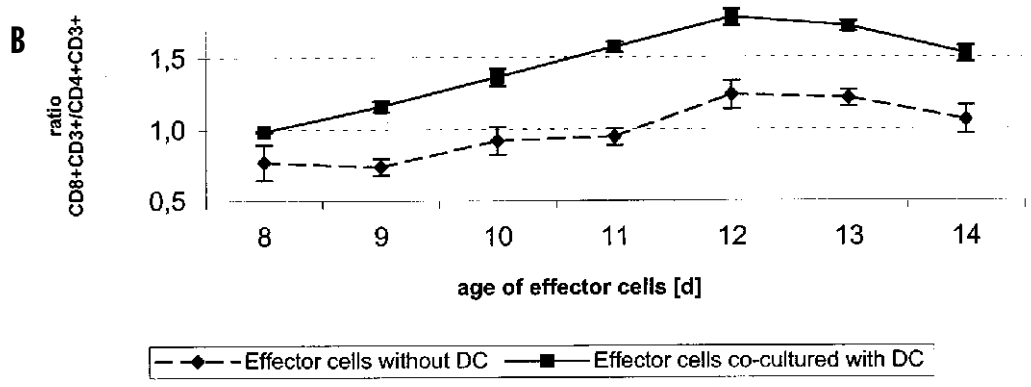


Figure 2. Phenotypic analysis of CIK cells. CIK lymphocytes were phenotyped on day +14 of culture using corresponding antibodies (A, left). Figures show representative data. The ratio between $CD8^+/CD3^+$ and $CD4^+/CD3^+$ cells over the course of time was determined by flow cytometry for either co-cultured or not co-cultured CIK cells (B, below). Co-culture was from days +7 to +14 with autologous DC. Data represent results from three separate experiments. Data are shown as mean ± standard error.



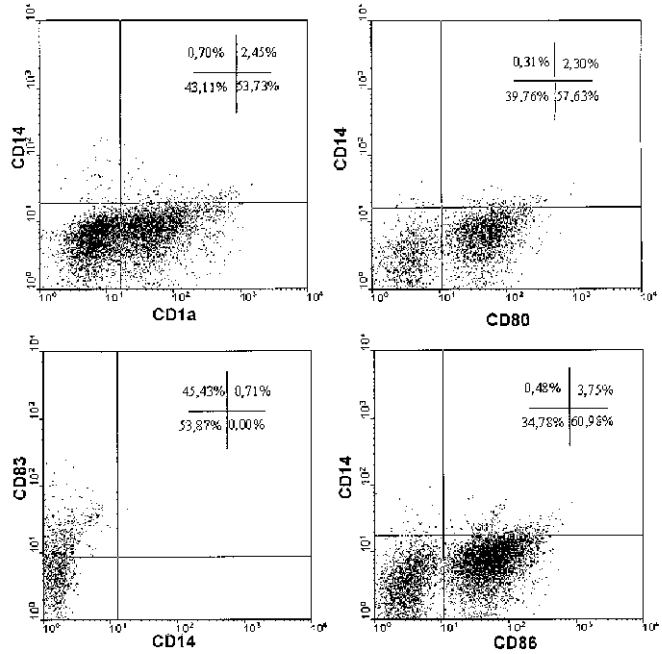


Figure 3. Flow cytometric analysis of DC. DC were phenotyped on day +14 of culture using corresponding antibodies. Figures show representative data.

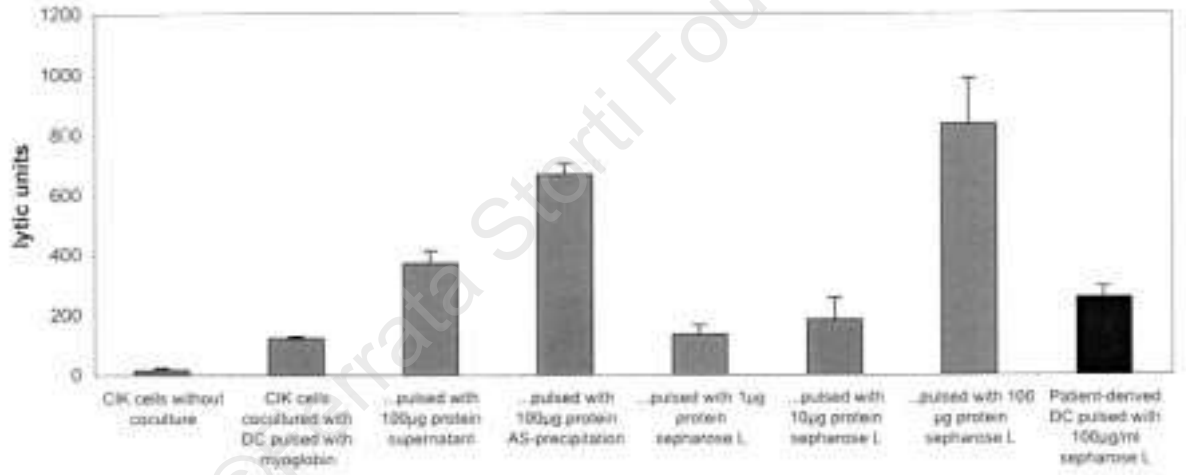


Figure 4. Cytotoxic activity of CIK lymphocytes after co-culture with idiotype-pulsed DC against MM cells. CIK lymphocytes from healthy donors or patients with MM were co-cultured from days +7 to +14 with autologous 7-day old DC cultures as described in the *Design and Methods* section. DC were stimulated either with irrelevant protein (myoglobin) or with idiotype from the target cell-line of different purification grade. Cytotoxic activity at various effector to target cell ratios was measured by the LDH-release assay and lytic units were calculated as described in the *Design and Methods* section. OPM-2 cells were used as targets. For comparison, CIK cells not co-cultured with DC were used as a control. Results represent data from at least three separate experiments. Data are shown as mean±standard error.

sepharose L (833±152 vs. 667±42 using AS precipitated supernatant vs. 370±36 lytic units for unpurified supernatant) (Figure 4). The increase of cytotoxic activity after co-culture with DC was significant (all $p < 0.05$). The cytotoxic activity of 833 lytic units was equivalent to 83.8±24.0% lysis at an effector to target ratio of 16:1 (data not shown).

Titration of various protein concentrations of sepharose L purified immunoglobulin showed a maximum at 100 µg/mL protein; higher protein concentrations did not lead to further enhancement (data not shown).

In further experiments, DC derived from patients with MM with elevated paraprotein serum levels

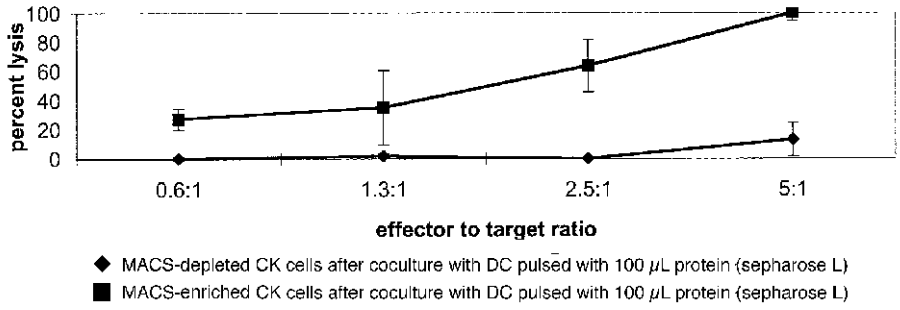


Figure 5. Cytotoxic activity of CIK cells after enrichment of IFN-γ secreting cells. CIK cells from healthy donors were enriched on day +14 by using the IFN-γ secretion assay after co-culture from days +7 to +14 with autologous DC. After enrichment, the effector cells were cultured for two further days. Cytotoxic activity at various effector to target cell ratios was measured by the LDH release assay. OPM-2 cells were used as targets. Results represent data from three separate experiments. Data are shown as mean±standard error.

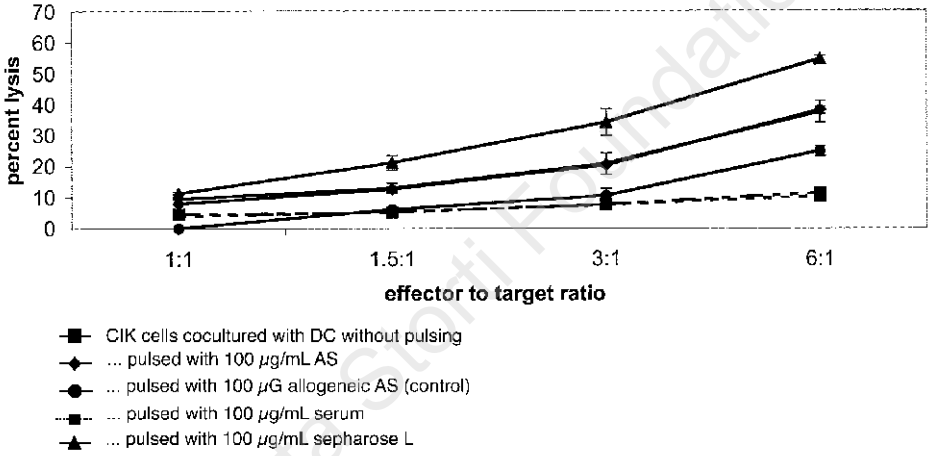


Figure 6. Cytotoxic activity of CIK lymphocytes from a patient with MM against autologous CD138+ cells. DC derived from a patient with MM with elevated paraprotein serum level were generated and tested for their capacity to stimulate CIK lymphocytes. CIK lymphocytes were co-cultured from days +7 to +14 with autologous DC pulsed with 100 µg/mL protein of different purification grade. Cytotoxic activity of CIK lymphocytes at various effector to target cell ratios was measured by the LDH release assay. Autologous CD138+ cells from bone marrow, enriched by the MACS technique, were used as targets.

were generated, pulsed with 100 µg/mL autologous protein and tested for their capacity to stimulate CIK lymphocytes. As targets, the allogeneic cell line OPM-2 was used. These cells secrete the same isotype as that secreted by the patients tested in this study (IgG). Similar to the results recorded with cells from healthy donors, pulsing DC with protein with higher purity led to a significant increase in cytotoxic activity of CIK lymphocytes (256±39 lytic units for sepharose L purified protein vs.7±5 lytic units for unpurified supernatant). In contrast, co-culturing with unpulsed DC led to no measurable cytotoxicity at the tested low effector to target ratios (Figure 4).

Comparing the lytic activity against OPM cells of CIK cells co-cultured with DC pulsed with idiotype from U266 cells (IgE) as control, and CIK cells co-cultured with unpulsed DC, we observed no increase (data not shown).

Analysis of cytotoxic capacity of IFN-γ producing immunologic effector cell cultures

Immunologic effector cells from healthy donors were co-cultured from days +7 to +14 with DC cultures pulsed with sepharose L purified idiotype of target cells. The activated cells were enriched using an IFN-γ secretion MACS technique on day +14. The percentage of activated

cells increased from below detectability to 1.2±0.4 % in the CIK populations. After MACS enrichment the purity of IFN-γ secreting cells ranged from 46 to 88%. Two days after enrichment, the cytotoxicity of effector cells was analyzed. After enrichment, co-cultured CIK cells were able to lyse 99.9±0.5% of OPM-2 cells at an effector to target ratio of 5:1. The depleted fraction showed a cytotoxic activity of 12.8±11.9% at the same surplus of effector cells (Figure 5). The increase of cytotoxic activity after MACS-enrichment was significant ($p = 0.02$).

Cytotoxicity of co-cultured immunologic effector cells against autologous primary cultures

When using a complete autologous model with primary culture of MM cells as targets, effector cells also showed cytotoxicity against target cells. MM cells from bone marrow were enriched using anti-CD138 microbeads. Effector cells co-cultured with autologous DC pulsed with sepharose L purified autologous serum showed an increase of lysis from 11.3% to 54.4% as compared to unpulsed DC at an effector to target cell ratio of 6:1 (Figure 5). Comparing DC pulsed with either autologous or allogeneic AS purified serum, autologous purified protein caused a higher increase in cytotoxicity (38.0% vs. 24.7%). Pulsing DC with autologous unpurified serum led to no increase in cytotoxicity compared to that of unpulsed DC (10.4% at an effector to target ratio of 6:1; Figure 6). The difference between cytotoxic activity after co-culture with serum-pulsed DC and sepharose L-pulsed DC was significant ($p = 0.047$).

Interestingly, investigating the same effector cells in a cytotoxicity assay against CD138 depleted bone marrow, we did not observe any autoreactive activity (*data not shown*).

Discussion

We observed cytotoxic activity of CIK cells against MM cells at low effector to target ratios. This effect could be strongly enhanced by co-culturing with specifically pulsed DC, and was also shown in a completely autologous setting against enriched $CD138^+$ cells from a patient with MM. At present, conventional therapy has a very limited curative potential for myeloma patients. Patients with myeloma and minimal residual disease may benefit from immunotherapeutic approaches. In this respect, numerous strategies of myeloma vaccination including idiotypic paraprotein, DC pulsed with paraprotein, whole myeloma cell vaccines and myeloma cell-DC fusions are under investigation.

Osterborg *et al.* immunized five myeloma patients by using M-component for repeated intradermal injections together with GM-CSF. All patients developed an idiotype-specific T-cell immunity.[22] Massaia *et al.* used Id-proteins conjugated to keyhole limpet hemocyanin (KLH) and low doses of s.c. GM-CSF or IL-2 in 12 patients in remission following high-dose chemotherapy and peripheral blood stem cell transplantation.[23] Id-specific T-cell proliferative responses were documented in two patients, whereas an Id-specific delayed-type hypersensitivity reaction was observed in eight of ten patients studied.

Numerous groups have examined DC-vaccines *in vitro* and in mouse models. For example, Osman *et al.* showed that blood-derived DC pulsed with myeloma cell extract activated specific CTL and induced them to kill autologous myeloma cells.[24] Most clinical studies demonstrated peripheral blood mononuclear cell- or T cell-proliferative responses, and side effects were mild or absent. Titzer *et al.* showed a biological response after vaccinating MM patients with idiotype-pulsed DC.[25] Valone *et al.* reported three complete remissions and three partial remissions in 13 patients with low tumor burden after vaccination with autologous idiotype-loaded DC and no tumor regression when patients had initially high tumor burden.[26-28]

With regard to data which predict the role of the innate immune system in MM,[14,15] we examined the cytotoxic activity of CIK cells[16-18] against MM. CIK cells have an enhanced cytotoxicity and a higher proliferation rate than LAK cells.[19,20] The higher lytic activity of CIK cells is mainly due to the higher proliferation of CD3 and CD56 double positive cells.[19] It has been shown that CIK cells are capable of eradicating an established human tumor in SCID mice.[29] Using the heterogeneous population of CIK cells, we tried to activate the adoptive and the innate arms of the immune system. T-cells can be primed in a MHC-restricted way by antigen-pulsed DC. The ability of NK- and NKT-cells to kill in a non-MHC restricted way could be enhanced by co-culture with DC.[30]

Although expressing an α, β T-cell receptor, the NKT-cell subpopulation ($CD3^+CD56^+$ cells) kill their targets through a non-MHC-restricted mechanism. Similar to NK-cells, CIK cells recognize killer inhibitory receptors (KIRs). Absence of KIRs on the cell surface of targets leads to lysis of target cells. Lysis of target cells is achieved via release of perforin and granzymes, which lyse the target cells and induce apoptosis.[31] In addition, Fas-mediated induction of apoptosis seems to play a minor role. In a first clinical trial, patients with metastatic dis-

eases were successfully treated with autologous IL-2-transfected CIK cells leading to clinical responses.[32]

Here, we showed the high alloreactive potential of CIK cells against MM cells at low effector to target ratios. CIK cells had a strong increase of lytic activity after co-culture with DC. This phenomenon seems to be caused by IL-12 secretion from DC and cell-cell interactions between the two populations (*unpublished data*). The cytotoxic effects could be further enhanced and directed in a specific manner by co-culturing with idiotype-pulsed DC. Not surprisingly, DC pulsed with idiotype of the highest purification grade (in this case, sepharose L-cleared idiotype after AS precipitation) mediated the highest cytolytic activity to co-cultured CIK cells. Comparing our data with those in reports using peripheral blood mononuclear cells co-cultured with idiotype-loaded DC, we found a significant increase in cytotoxic activity with a shift to much lower effector to target ratios.[15,33,34] We speculate that this phenomenon is caused by the high non-specific basic cytotoxic activity of our CIK cells.

Using effector cells from patients with MM we were able to reproduce our data with effector cells from healthy donors, but the cytotoxic potential was weaker, reflecting the reduced immune status of the patients. A further shift to low effector to target ratios was achieved by using MACS enriched effector cells. The cytokine secretion MACS assay allows the enrichment of activated cells. These cells represent only a small fraction of the whole population, but they have an extremely high cytotoxic potential for immunotherapeutic approaches against MM. Further studies are required to expand these cells. Furthermore, preliminary data shown here demonstrate the possibility of inducing reactivity against autologous tumor cells, but not against healthy tissue. In conclusion, CIK cells showed lytic activity against MM cells. This activity was enhanced by co-culturing with antigen-specific pulsed DC. CIK cells co-cultured with DC pulsed with idiotype may have a major impact on immunotherapeutic protocols for MM patients.

Contributions and Acknowledgments

AM designed the project and wrote the paper. SR analyzed the data and helped to write the paper. MvL-T, PB and FS acted in an advisory capacity and critically read the manuscript. TS and IGHS-W helped with the conception of the project and critically read the manuscript. They all approved the final version of the manuscript. We thank Essex, Munich, Germany for their kind gift of IL-4.

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Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

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Potential implications for clinical practice

CIK cells co-cultured with DC pulsed with idiotype may have a major impact on immunotherapeutic protocols for MM patients.

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