**Increased effect of IMiDs by addition of cytokine-induced killer cells in multiple myeloma**

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**Abstract**

Immunomodulatory drugs (IMiDs), such as thalidomide, lenalidomide and pomalidomide, represent the basic principle of multiple myeloma treatment. However, the development of resistance is a limiting factor. Over the last years, the efficient application of cytokine-induced killer (CIK) cells has been reported as an alternative strategy to treat hematological neoplasms. In this study, we tested for a potential synergistic effect by combining the IMiDs thalidomide, lenalidomide and pomalidomide with CIK cells in different myeloma cell lines *in vitro*. Myeloma cells tested with CIK cells were significantly reduced. In the combination, myeloma cells were significantly reduced compared with cells only tested with IMiDs but not to the cells tested with CIK cells. Otherwise, the number of CIK cells was significantly reduced when treated with IMiDs. Because IMiDs are active in patients with myeloma, these results lead to the expectation that combination of IMiDs and CIK cells achieve better results in the treatment of multiple myeloma compared with the single use of IMiDs. Therefore, further examinations in an *in vivo* setting are necessary to have a closer look on the cellular interactions. Copyright © 2015 John Wiley & Sons, Ltd.

**Keywords:** multiple myeloma; cytokine-induced killer cells; immunomodulatory drugs; immunotherapy

**Introduction**

Multiple myeloma (MM) is a hematological malignancy, based on monoclonal expansion of plasma cells in the bone marrow [1]. With the introduction of thalidomide (Thal) and other immunomodulatory drugs (IMiDs) such as lenalidomide (Lena) and pomalidomide (Pom), new options for the treatment of MM were given [2–4]. Although the molecular mechanism of those IMiDs remains unclear, several direct and indirect anti-myeloma effects have been described such as anti-angiogenic, anti-proliferative, anti-inflammatory, pro-apoptotic and immunomodulatory effects [5]. Nevertheless, only 30% of patients with MM respond to single used IMiDs [6], and the development of resistances leads to further complications [7]. Thus, until today, MM remains an incurable disease.

The immunomodulatory effects of IMiDs, like stimulation of T cells [8] and an enhanced lytic activity of natural killer (NK) and natural killer T (NKT) cells [9], lead to the expectation that the combination of IMiDs and adoptive immunotherapy with cytokine-induced killer (CIK) cells might be a new option in the treatment of MM.

CIK cells are a heterogeneous cell population consisting predominantly of CD3⁺CD56⁺ cells and in minor amounts of CD3⁻CD56⁻ T cells as well as CD3⁻CD56⁺ NK cells [10]. They possess cytolytic capacities towards different types of tumour cells *in vitro* and *in vivo* including MM cells [11–13]. This cytotoxicity is mediated via a non-major histocompatibility complex (MHC)-restricted mechanism [14].

In this study, we investigated the cytotoxic efficiency of CIK cells in combination with IMiDs towards different myeloma cell lines *in vitro*; furthermore, we examined the effect of IMiDs on CIK cells.

**Material and methods**

**Cell lines and culture conditions**

The myeloma cell lines OPM-2, U-266 and KMS-11 [all obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Braunschweig, Germany] were cultured in RPMI-1640 medium (PAA, Cölbe, Germany) supplemented with 10% heat-inactivated fetal calf serum (FCS) (life technologies, Darmstadt, Germany), 100 U/ml penicillin (life technologies) and 100 μg/ml streptomycin (life technologies). The myeloma cell line KMS-12 PE (DSMZ) was cultured in RPMI-1640 medium with 20% heat-inactivated FCS.
The control cell line CCD-18Co (ATCC, Wesel, Germany) (human colon fibroblasts) was cultured in Eagle’s Minimum Essential Medium (ATCC) consisting of 10% heat-inactivated FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were incubated at 37°C in humidified 5% CO₂ atmosphere.

**Generation of CIK cells**

CIK cells were generated as previously described¹⁴. In short, non-adherent Ficoll-separated (Lymphoprep, PAA) human peripheral blood mononuclear cells from healthy donors were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FCS, 25 mmol/l Hepes (PAA), 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were grown at a density of 5 × 10⁶ cell/ml. One thousand units per milliliter(U/ml) recombinant human interferon gamma (rh IFN-γ) (ImmunoTools, Friesoythe, Germany) was added at day 0. After 24 h, 300 U/ml rh interleukin-2 (rh IL-2), 100 U/ml IL-1β (both ImmunoTools) and 50 ng/ml anti-CD3 (eBioscience, Frankfurt, Germany) were added. Every 3 days, further 300 U/ml rh IL-2 was added; after 14 days, the CIK cells were matured and ready for use.

**Drugs**

The following drugs were used in this setting: Thal (Sigma-Aldrich, München, Germany), Lena (Selleck, München, Germany) and Pom (Sigma-Aldrich). All drugs were used at different concentrations for 24–72 h.

**MTT assay**

An MTT assay was performed to measure the cytotoxicity of the different drugs and the CIK cells. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is a colorimetric alternative to the ^51^Cr release assay, whereby the yellow MTT is reduced to purple formazan by viable cells [15].

In brief, 1–10 × 10⁴ cells were plated in triplicates in flat bottomed 96-well plates and co-incubated with various concentrations of IMiDs and CIK cells over 24–72 h before MTT reagent was added. After 3 h of incubation, the plates were centrifuged for 4 min, 1200 rpm. The supernatant was

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**Figure 1.** Effect of the IMiDs Thal, Lena and Pom on the viability of the myeloma cell lines OPM-2, U-266, KMS-11 and KMS-12 PE. Cells (1 × 10⁵) were cultured for 48 h with different concentrations. Thal: 0.1 μM, 1 μM, 10 μM, 100 μM and 400 μM; Lena: 0.1 μM, 1 μM, 10 μM, 100 μM and 200 μM; and Pom: 0.1 μM, 1 μM, 10 μM and 100 μM. Cell viability was measured via MTT assay. Results represent data from three separate experiments. Data are shown as mean ± standard deviation (SD). (p < 0.05, two-way ANOVA)
abolished, and 80μl of MTT lysis buffer (isopropanol, HCl) was added. After shaking the plates for 10 min at 700 rpm, colorimetric analysis was performed on a multiwell scanning spectrophotometer (FluoStar Optima, BMG Labtech, Ortenberg, Germany) at 492 nm.

Enzyme-linked immunosorbent assay (ELISA)

For measurement of interferon gamma secretion, a sandwich ELISA was performed (Thermo Fisher Scientific Inc., Rockford, USA). Myeloma cells of $2 \times 10^5$ were

Figure 2. Cytotoxic effect of CIK cells on myeloma cell lines OPM-2, U-266, KMS-11 and KMS-12 PE and control cells CCD-18Co. Cells were cultured for 24 h at different effector: target ratios of 1:5, 1:2 and 1:1. Cell viability was measured via MTT assay. Results represent four separate experiments for OPM-2, U-266, KMS-11 and CCD-18Co and three separate experiments for KMS-12 PE. Data are presented as mean ± SD. ($p < 0.05$, one-way ANOVA)
plated in triplicates in 96-well plates and co-incubated with Thal, Lena or Pom. After 24 h, 1 × 10^5 CIK cells were added. After another 24 h, the plates were centrifuged for 5 min, 1100 rpm. Then 50 μl supernatant was transferred to an antibody coated ELISA plate. Thereafter, the assay was continued according to the manuals instructions. Absorbance was measured with an ELISA reader at 450 nm (Glomax multidetection system, Promega, Mannheim, Germany).

Flowcytometric analysis

Phenotypes of treated and untreated CIK cells were determined by fluorescence-activated cell sorting analysis (FACS Canto II, BD Bioscience, San Jose, USA). Cells were stained with the monoclonal antibodies (mAb) anti-human CD45 PerCP-Cyanine5.5, anti-human CD3 FITC, anti-human CD56 APC, anti-human NKG2D (CD314) PE (eBioscience, Frankfurt, Germany), anti-human CD25 PE and anti-human CD69 PE (BioLegend, Fell, Germany). FlowJo Data Analysis Software (Ashland, USA) was used for data analysis.

Statistics

GraphPad Prism (La Jolla, USA) was used for statistical analysis. One-way and two-way analysis of variance (ANOVA) with Bonferoni posttest was performed to analyze statistical significance. The p-values < 0.05 were considered as significant.

Results

Effect of Thal, Lena and Pom on myeloma cell lines

The myeloma cell lines OPM-2, U-266, KMS-11 and KMS-12 PE were cultured with increasing concentrations of Thal (1 μM, 10 μM, 100 μM, 200 μM and 400 μM), Lena PE and anti-human CD69 PE (BioLegend, Fell, Germany). FlowJo Data Analysis Software (Ashland, USA) was used for data analysis.

Figure 3. Combined effect of IMiDs and CIK cells on viability of myeloma cells U-266, KMS-11 and KMS-12 PE. Cells were cultured with different concentrations of IMiDs (Thal 100 μM, Lena 100 μM and Pom 10 μM) after 24 h (A, B and C)/72 h (D, E and F) medium was removed and cells were cultured for another 24 h at different effector: target ratios (1:5, 1:2 and 1:1 for A, B and C and 1:5, 1:7.5 and 1:10 for D, E and F) of CIK cells. Cell viability was measured via MTT assay. Results represent data from three different buffy coats for U-266 and KMS-12 PE and two different buffy coats for KMS-11 (in E also three buffy coats). Data are shown as mean ± SD. (p < 0.001, two-way ANOVA)
(0.1 μM, 1 μM, 10 μM, 100 μM and 200 μM) or Pom (0.1 μM, 1 μM, 10 μM and 100 μM) over 24–72 h (Figure 1). Nevertheless, none of them had a significant effect on the viability of myeloma cells in vitro.

**Effect of CIK cells on myeloma cell lines**

CIK cells were co-cultured with myeloma cell lines OPM-2, U-266, KMS-11 and KMS-12 PE and the control cell line CCD-18Co for 24 h (Figure 2). Therefore, CIK cells of four (three in KMS-12 PE) different buffy coats were used with different effector to target ratios (1:5, 1:2 and 1:1). CIK cells reduced significantly the viability of myeloma cells in vitro. The observed effect was in correlation to the different effector to target ratios. However, the viability of CCD-18Co was also significant reduced by CIK cells.

**Combination of Thal, Lena and Pom with CIK cells**

To investigate a synergistic effect of IMiDs and CIK cells, myeloma cell lines U-266, KMS-11 and KMS-12 PE were first cultured with Thal (100 μM), Lena (100 μM) or Pom (10 μM) for 24 or 72 h. After 24 h, the whole medium was changed and the cells were cultured for another 24 h with CIK cells at different effector to target ratios (1:5, 1:2 and 1:1) (Figure 3). Cells tested for 72 h with IMiDs were co-cultured with CIK cells for 24 h with different effector to target ratios (1:10, 7:5:1 and 5:1). The viability of myeloma cells treated with IMiDs (24 h) and CIK cells was significantly lower compared with the viability of myeloma cells co-cultured only with IMiDs (Figure 4). Myeloma cells only cultured with CIK cells showed no significance towards the combined treated cells. The control cells CCD-18Co showed the same results as the myeloma cells (data not shown). In summary, no synergistic effect of IMiDs and CIK cells was traceable in this setting.

In another setting, myeloma cells (U-266, KMS-11 and KMS-12 PE) were cultured for 72 h with previously described IMiDs. Again, whole medium was changed, and CIK cells with even lower effector to target ratios (1:5, 1:7, 5 and 1:10) were added for 24 h (Figure 3).

In all three cell lines, there was no significant difference in the samples treated with Thal. Cells treated with Lena showed different results. In U-266, there were no significant differences. In KMS-11, cells treated with CIK cells (1:10) showed significantly lower viability compared with cells only treated with Lena. KMS-12 PE cells treated with Lena exposed significantly lower viability compared with cells treated with CIK cells.

Samples treated both with Lena and CIK cells (1:5) showed lower viability compared with cells only treated with CIK cells (1:5).

Cells treated with Pom showed the same results as cells treated with Lena.

**Interferon γ secretion in the combination of IMiDs and CIK cells on myeloma cells**

Myeloma cell lines U-266, KMS-11 and KMS-12 PE were first incubated with Thal (100 μM), Lena (100 μM) or Pom (10 μM), after 24 h, the whole medium was changed and CIK cells were added at an effector to target ratio of 1:2. After another 24 h, interferon γ secretion was measured by a sandwich ELISA (Figure 5).
In all three myeloma cell lines, a stable IFN-γ secretion was measured whether treated with IMiDs or not. CIK cells treated with IMiDs acted in the same way, but IFN-γ secretion in total was higher.

In all three cell lines, interferon γ secretion was significantly higher in the combined samples compared with the samples treated only with IMiDs. Samples treated with CIK cells showed a significantly higher IFN-γ secretion in the cell line KMS-11 compared with the IMiD samples, but significance was not as high as in the combined samples.

In KMS-11, the combination with Pom also showed significantly higher IFN-γ secretion compared with untreated CIK cells and compared with the combination with Thal.

In KMS-12 PE, the combination with Lena displayed a significantly higher secretion of IFN-γ compared with untreated CIK cells. Overall secretion of IFN-γ in KMS-12 PE cells treated with CIK cells or the combination of CIK cells an IMiDs was much higher compared with the other cell lines. KMS-12 PE cells treated or untreated with IMiDs showed the same INF-γ secretion as the other cell lines.

**Effect of Thal, Lena and Pom on viability of CIK cells**

CIK cells of four different buffy coats were tested with Thal, Lena (both 100 µM) or Pom (10 µM) for 24 h (Figure 6). All three IMiDs decreased the viability of CIK cells significantly. Pom had a significantly higher effect on CIK cells than Thal and Lena.

**Effect of IMiDs on phenotypical determination of CIK cells**

CIK cells of five different buffy coats were treated with Thal (100 µM), Lena (100 µM) or Pom (10 µM). After 24 h, treated and untreated cells were stained with mAb CD45, CD3, CD56, CD25, CD69 and NKG2D.
CD3 and CD56 were used to characterize the different CIK cell subsets (Figure 7). The flow cytometric analysis did not show any difference between these cell subsets in treated and untreated cells. CD3+CD56+ cells represent the main group of cells followed by CD3+CD56− and CD3−CD56−.

CD 25, CD 69 and NKG2D were used as markers for the activation of CIK cells. CIK cells of various buffy coats showed different results but same tendencies.

CD 25 in general was expressed only by a small number of cells. Expression of CD69 was higher especially in CD3+CD56+ cells. NKG2D showed high expression over all three cell subsets, most of all in CD3+CD56+ cells.

In cells marked with CD25, treatment with Lena and Pom caused an increased frequency of CD3+CD56+ and CD3−CD56− cells. Cells treated with Pom even showed a slight enhancement of CD3−CD56− cells. Thal had the same effect but in an attenuated form.

Cells marked with CD69 or NKG2D showed different results. Treatment with Thal, Lena or Pom led to slightly decreased frequencies of CD3+CD56+, CD3−CD56− and CD3−CD56+ cells compared with untreated controls (Figure 7).

**Discussion**

IMiDs are already widely used in the treatment of MM. Its anti-myeloma effects *in vivo* have been recently shown. In clinical studies, the combination of Thal/dexamethasone and Lena/dexamethasone achieved partial response rates of 61.2 and 80.3 % respectively, with tolerable side effects [16]. However, we observed a slightly lower anti-myeloma effect of IMiDs *in vitro*. This might be explained by the complex mechanisms of action of IMiDs. The direct antitumour effect just represents a small part of the whole potentiality of IMiDs. Effects on the microenvironment of MM or immunomodulatory effects cannot be revealed by *in vitro* testing. Nevertheless, chemotherapy with IMiDs remains one of the backbones in the treatment of MM.

![Figure 7](http://example.com/figure7.png)

**Figure 7.** (A) Flowcytometric analysis was used to identify untreated and treated [thal (100 μM), lena (100 μM) and pom (10 μM) for 24 h] CD3+CD56+ CIK cells. Data show representative dot plots. Furthermore, flow cytometry was used to determine the activation markers CD 25, CD69 and NKG2D. Data are representative for all activation markers. (B) Distribution of different phenotypes in treated and untreated CIK cells. Cells were treated for 24 h with Thal (100 μM), Lena (100 μM) or Pom (10 μM). Results represent CIK cells of five different buffy coats. Data are shown as mean ± SD (p < 0.001, one-way ANOVA). (C) Expression of CD25, CD69 or NKG2D on CD3+CD56+ CIK cells. Cells were either untreated or treated with Thal100 μM, Lena 100 μM or Pom 10 μM for 24 h. Data represent one generic buffy coat.
A different approach is the use of CIK cells in patients with MM [17]. We investigated the anti-myeloma effect of CIK cells from healthy donors on different myeloma cell lines in vitro. It was recently shown that CIK cells have the capability to lyse myeloma cells when previously co-cultured with dendritic cells (DCs) [11]. In this study, we demonstrate the anti-myeloma effect of untreated CIK cells on MM cells at very low effector to target ratios. CIK cells were able to lyse MM cells to a significant extent even when tested with an effector to target ratio of 1:2. The lytic effect of CIK cells on tumour cells is mediated via non-MHC-restricted, NK-like recognition. The expression of NK cell receptors, like NKG2D, seems to play an important role in the recognition of myeloma cells [18]. Nevertheless, other studies showed that CIK cells keep their TCR-mediated specific cytoxicity during maturation [19]. In summary, these complex mechanisms, how CIK cells eliminate tumour cells, still remain unclear.

Furthermore, we investigated the anti-myeloma effect of IMiDs and CIK cells when applied in combination. The anti-myeloma effect in the combination is significantly higher compared with the effect of only IMiDs. In contrast to this, other data show significant reduction of CIK cells when tested with IMiDs. These results lead to the possible mechanisms how IMiDs interact with CIK cells. The immunomodulatory effect of IMiDs occurs on different levels, one is the costimulation of T cells. Costimulation is mediated via T-cell receptor but also requires further signals of antigen presenting cells. Activation of T cells leads to increased proliferation and production of IL-2 and INF-γ [8,20]. Increased production of IL-2 and INF-γ induces proliferation of NK cells and improves their lytic capability towards myeloma cells [21,22]. Another effect is the enhancement of NKT cells. In the presence of Lena, DC-mediated NKT cell expansion as well as INF-γ production is increased and causes further activation and proliferation of NK cells [9]. Therefore, the immunomodulatory effect of IMiDs affects at least most cell types in the heterogeneous population of CIK cells. Nevertheless, it has to be considered that the outcome of the study is strongly influenced by other variables like cell–cell interaction or endogenous mediators.

An additional feature of IMiDs is their anti-inflammatory potential. They are able to block the transcription factor NF-κB and thereby the expression of inflammatory genes that lead to a reduced secretion of inflammatory cytokines [23]. In our setting, this may cause a restricted expansion of the CIK cells full activity.

It has been already proposed that IMiDs have an effect on regulatory T cells (Tregs). Different studies have shown conflicting outcomes. On the one hand, IMiDs inhibited proliferation of Tregs [24]. Other studies showed an increased number of Tregs in patients treated with IMiDs [25]. The impact of IMiDs on the Treg population in CIK cells is another interesting point that needs to be further investigated. The anti-inflammatory effect of IMiDs, as well as the interaction with Tregs, may cause the decreased number of CIK cells we observed in this study.

In summary, the combination of IMiDs and adoptive immunotherapy seems to be a promising alternative for treatment of MM. Because of the complex mechanisms of interaction, it is essential to detect the right timing for the application of CIK cells in an experimental setting, where the synergistic effects prevail over negative interactions. This opens new questions that have to be addressed in an in vivo model.

Conflict of interest

All authors declare no conflict of interest.

References


