PD-1 blockade enhances cytokine-induced killer cell-mediated cytotoxicity in B-cell non-Hodgkin lymphoma cell lines

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Abstract. The clinical utility of immune checkpoint inhibitors, such as programmed cell death protein 1 (PD-1) and programmed death-ligand 1 (PD-L1) inhibitors used alone or in combination with other therapies, is currently gaining attention. In this particular scenario, the inclusion of cytokine-induced killer (CIK) cells has proven to be a novel therapeutic approach. CIK cells have shown anticancer activity in various hematopoietic malignancies, but their defined cytotoxicity in B-cell non-Hodgkin lymphoma (B-NHL) remains to be fully elucidated. The present study investigated the role of PD-1/PD-L1 blockades on the cytotoxic efficacy of CIK cells primarily in B-NHL cell lines. The current analysis revealed that CIK cells prompted cytotoxicity against B-NHL cell lines (DAUDI and SU-DHL-4), and a significant increase in PD-L1 expression was observed when CIK cells were co-cultured with B-NHL cells. Additionally, a combination of PD-1 and PD-L1 antibodies with CIK cells significantly decreased cell viability only in DAUDI cells. Furthermore, IFN-γ elevation was observed in both cell lines treated with CIK alone or with PD-1 antibody, but this tendency was not observed for PD-L1. Since PD-1 can suppress immune inactivation, whereas CD40L can promote it, the effects of CD40L blockade were also examined; however, no significant changes in cell viability were observed. Overall, the present *in vitro* data suggested that CIK cells exerted a cytotoxic function in B-NHL cells, and a combination of PD-1 inhibitors with CIK cells may provide a potential therapeutic option for this type of lymphoma. Nevertheless, *in vivo* experiments are warranted to undermine the extent to which PD-1 inhibitors may be used to enhance the antitumor activity of CIK cells in B-NHL.

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Introduction

Over the years, several therapeutic advances have been made to cure malignant lymphomas, especially, non-Hodgkin's lymphoma (NHL) which is much less predictable compared to Hodgkin's lymphomas. Of importance, diffuse large B-cell lymphoma (DLBCL) represents the most common NHL subtype, which poses a major clinical challenge due to its heterogeneity, variable efficacy, multiple side effects, and frequent relapses.

Recent studies using programmed cell death 1 (PD-1) blockade have shown some promising outcomes in the phase I trial of B-cell non-Hodgkin's lymphoma (B-NHL) (1,2). PD-1 and its ligand (PD-L1/L2) have also been pronounced as diagnostic and prognostic determinant in lymphomas (3). This can be evident from immunohistochemistry-based studies where the variable expression of PD-L1 has been observed in classical Hodgkin's lymphoma (87-100%) (4), diffuse large B-cell lymphoma (11-31%) (5,6) and Burkitt's lymphoma (0%) (7).

Increasing the clinical utility of PD blockades alone or with combination therapies is currently gaining momentum, and in this context, cytokine-induced killer (CIK) cells are emerging as a new potential partner. CIK cells, as heterogeneous subset of *ex vivo* expanded T lymphocytes, exhibit cytotoxicity toward tumor cells thus contribute to prolong the survival in cancer patients (8-10). The use of autologous and allogeneic CIK cells in the clinical trials of acute myeloid leukemia, chronic myeloid leukemia, and chronic lymphocytic leukemia had already demonstrated an innovative clinical perspective (11-13). However, the cytotoxicity of CIK cells against B-NHL has not been fully elucidated. Also, the efficacy of combining PD-1 blockade with CIK cells in B-NHL (*in vitro* or *in vivo*) remains unclear.

Therefore, we aimed to investigate the cytotoxic potential of CIK cells in B-NHL cell lines and further elucidate the

relative contribution of PD-1/PD-L1 inhibitors towards CIK-mediated antitumor immune response. Given that PD-1 can suppress immune inactivation, whereas CD40L can promote it, we also examined the effects of CD40L blockade under the same experimental conditions.

Materials and methods

Cell culture and generation of CIK cells. CIK cells were cultured in RPMI-1640 medium (PAN Biotech) supplemented with 10% heat-inactivated FBS, 1% penicillin/streptomycin, and 2.5% HEPES (Gibco; Thermo Fisher Scientific, Inc.). Human B-lymphoblast cell lines: DAUDI (Burkitt's lymphoma, DSMZ) and SU-DHL-4 (diffuse large B-cell lymphoma, ATCC) were used in the study. RPMI-1640 medium supplemented with 10% heat-inactivated FBS (Sigma-Aldrich Chemie GmbH) and 1% penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.) was used to culture tumor cells (37˚C, 5% CO_2). All cell lines were mycoplasma negative as confirmed by MycoAlert™ mycoplasma detection kit (Lonza).

CIK cells generation and expansion. For CIK cell generation, peripheral blood mononuclear cells (PMBCs) were derived from buffy coats of healthy volunteers received from the Blutspendedienst at the University Hospital Bonn. Approval of the ethics committee of the University Hospital Bonn was obtained, including signed informed consent from the volunteers. CIK cells were generated as previously described protocol (14). Briefly, a standard gradient density centrifugation using Pancoll (Pan-Biotech) was performed. Subsequently, the PBMC layer containing the lymphocytes was removed and washed one time with PBS/0.4% EDTA, and treated with an erythrocyte lysis buffer (Biolegend). With sequential addition of IFN-γ 1,000 IU/ml on day 0, and 50 ng/ml monoclonal antibody against CD3 (anti-CD3 mAb) and 100 IU/ml interleukin-1β (IL-1β) and 600 IU/ml interleukin-2 (IL-2) on the next days, the cells were expanded.

PD-L1 and PD-L2 cell surface expression detection by flow cytometry. CIK cells at day 14 were cultured with $5x10^5$ CFSE-labeled DAUDI and/or SU-DHL-4 cells, primarily, at E/T (effector cell-CIK cell/target cell-tumor cell) ratio of 5:1 for 24 h. PE-conjugated anti-PD-L1, BV421-conjugated anti-PD-L2, and PerCP-conjugated 7-AAD (BD Bioscience) were used for the flow cytometric detections. CIK cells were generated from different donors (n=3).

Cell viability assays. Despite the different detection principles for cytotoxicity evaluation, we used two parallel approaches (MTT assay and CCK-8 cell viability assay) to avoid any misleading results. Both assays were performed, as described by the manufacturer (MTT assay: Sigma-Aldrich; CCK-8 cell viability assay: Dojindo). In MTT assay, the detection was measured at 560 nm using a plate reader (BMG Labtech) and the data were normalized to the amount of CIK cells used. While, the OD measurements of CCK-8 assay were taken at 450 nm. Untreated tumor cells (as negative control) and CIK cells co-cultured with tumor cells (as positive control) at different E/T ratios of 0.1:1, 1:1, 3:1 were used in the experimental setup.

Blockade of receptor-ligand interaction. To block the PD-1 receptor present on CIK cells, a polyclonal human PD-1 antibody (R&D Systems) was used and CIK cells were incubated with 3-12 μg/ml anti-rhPD-1 antibody (for 2 h) before incubation with the tumor cells. Similarly, a polyclonal human B7-H1/PD-L1 antibody (R&D Systems) was used to block PD-L1 expressed by the tumor cell lines. Here again, tumor cells were incubated with 1-5 μg/ml anti-B7-H1/PD-L1 for 2 h before incubation with CIK cells. In case of CD40L expressed on the tumor cells, a human CD40L/TNFSF5 monoclonal antibody (R&D Systems) was used and the tumor cells were incubated with 0.03-0.1 μg/ml anti-rhCD40L for 2 h before incubation with CIK cells.

Human IFN-γ ELISA. To determine the amount of IFN-γ produced by CIK cells, a Duoset® Human IFN-γ ELISA kit (R&D Systems) was used, as described by the manufacturer. The final measurements were performed at 450 and 540 nm wavelengths and the data were analyzed using MARS data analysis (BMG Labtech).

Statistical analysis. Unpaired, two-tailed Student's t-test was performed to evaluate the effect of CIK incubation with tumor cells on PD-L1/PD-L2 expression compared with the untreated tumor group at different ratios. One-way ANOVA with Turkey's post-hoc test was performed to compare multiple groups. A 4-PL non-linear regression model was used to calibrate the data for the ELISA assay. Statistical analysis was performed using Prism software (GraphPad Prism version 5.0 f) and a value of $P<0.05$ was considered significant ($^{*}P<0.05$; $^{**}P<0.005$; $^{***}P<0.001$).

Results

CIK cells displayed cytotoxicity towards B-cell non-Hodgkin leukemia cells. In both cell lines, very low levels of PD-L1 (DAUDI: 4.62±0.39%; SU-DHL-4: 1.97±0.37%) and PD-L2 (<2%) were observed. However, a significant increase in PD-L1 (DAUDI: 48.13±2.01%; SU-DHL-4: 96.57±0.47%) was observed when CIK cells were co-cultured with them (24 h, E/T ratio of 5:1) (Fig. 1A and 1B). Noticeably, PD-L2 levels remained unchanged.

Additionally, we determined the percentage of viable cells at varying target cell-to-effector cell ratios (E/T ratios: 0.1:1, 1:1, 3:1) by using the MTT assay. We found that DAUDI cells were more viable compared to SU-DHL-4 (Fig. 2A and B). Notably, at E/T ratio of 1:1, the viability of DAUDI cells was found to be reduced (by 35%) compared to the positive control, while it was decreased severely (by 50%) in case of SU-DHL-4. A further increase in the E/T ratio to 3:1 resulted in a continued decrease in cell viability of SU-DHL-4 by >70%.

Enhanced cytotoxicity of CIK cells in B-NHL induced by anti-PD-1 antibodies. To investigate the amount of antibody that could effectively block PD-1 and PD-L1 we performed a series of titrations with them and examined the cell viability

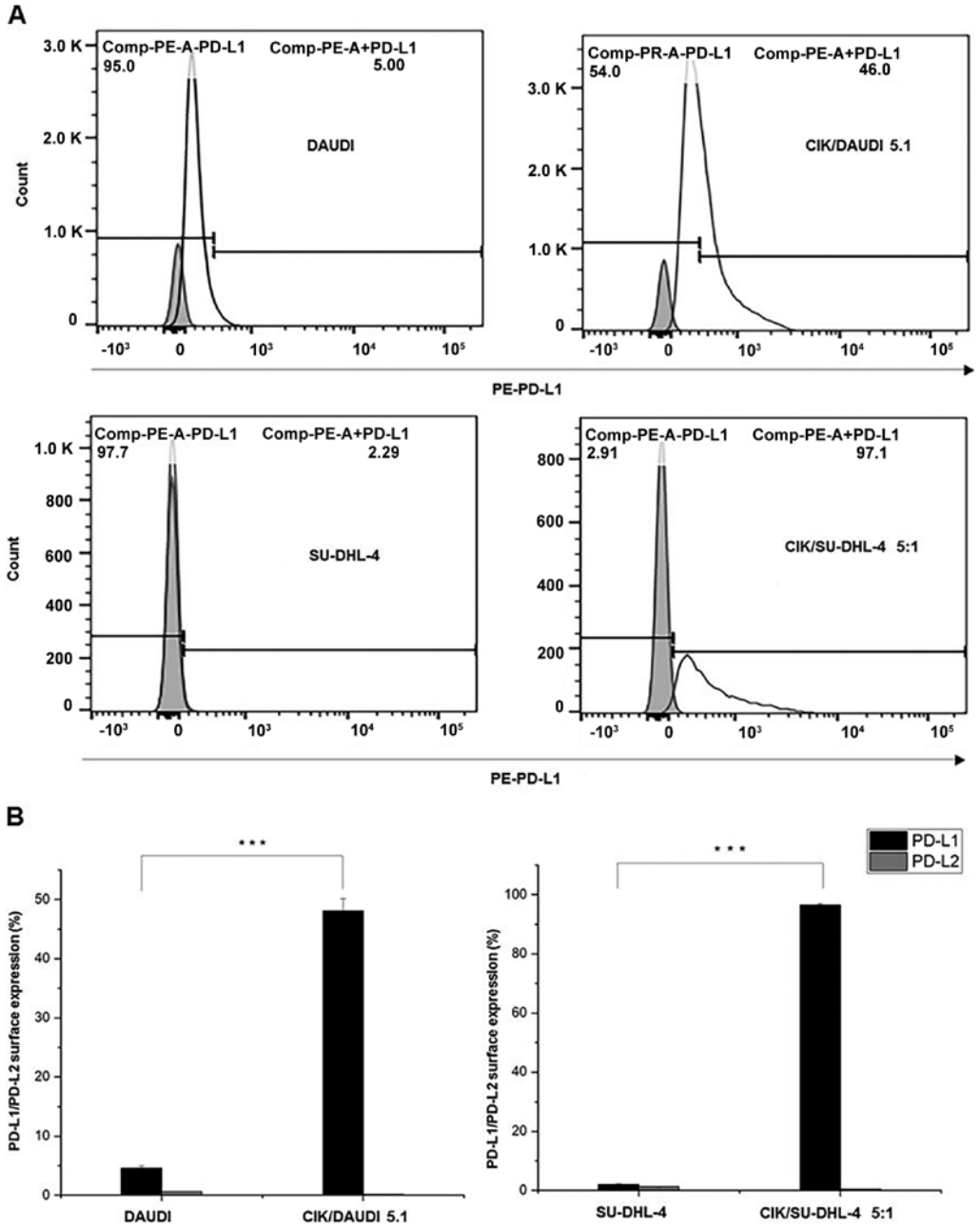

Figure 1. Flow cytometry evaluations of PD-L1 and PD-L2 expression in DAUDI and SU-DHL-4 cells with or without addition of CIK cells. (A) Histograms plotted on 7AAD-negative live cells indicating PD-L1 and PD-L2 expression (white) with the corresponding isotype control (gray). (B) Bar graphs of the obtained values. Analyses were performed on different donors (n=3), and values are displayed as the mean ± SD. Student's t-test, ***P<0.001. PD-L1/2, programmed death-ligand 1/2; CIK, cytokine-induced killer.

using CCK-8 assay. The use of 5 μg/ml PD-L1 antibody significantly decreased the cell viability (~60%) compared to positive control in DAUDI cells (after being cultured with CIK cells for 24 h) (Fig. 3A). 12 μg/ml PD-1 was required to achieve the comparable results (Fig. 3B).

Notably, the cell viability in both cell lines (DAUDI and SU-DHL-4) was severely impacted when co-cultured with PD-1 blockade-activated CIK cells (Fig. 4A, B). For instance, the concentration of 12 μg/ml PD-1 antibody led to a significant decrease in cell viability in DAUDI (~38%) and SU-DHL-4 (~50%) cells. To mention, no significant difference was observed after treatment with PD-L1 (5 μg/ml) and/or CD40L (0.1 μg/ml) antibodies. However, it cannot be excluded that the high concentrations of CD40L (~1 μg/ml) may exert severe cytotoxic effects. Interestingly, PD-1 combined with PD-L1 blockade showed significant differences only when CIK cells were co-cultured with DAUDI cells.

Blocking of the PD-1 receptor present on CIK cells enhances the secretion of IFN-γ. To evaluate the efficiency of PD-1 and PD-L1 blockade, we performed an IFN-γ ELISA assay and observed a significant increase in IFN-γ secretion in both cell lines treated with CIK alone and/or with PD-1 antibody (Fig. 5). In contrast, this tendency was not observed for PD-L1. Similarly, we also examined the effects of CD40L blockade but did not observe any significant changes.

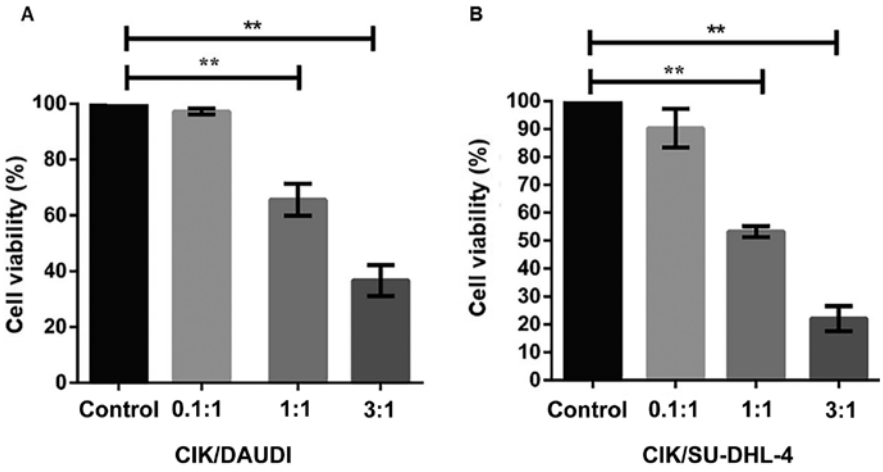

Figure 2. Cell viability in DAUDI and SU-DHL-4 cells at different E/T ratios. Cell viability in (A) DAUDI and (B) SU-DHL-4 cells co-cultured with CIK cells at different E/T ratios was measured using the MTT assay. **P<0.005. CIK, cytokine-induced killer; E/T, effector/target cells ratio.

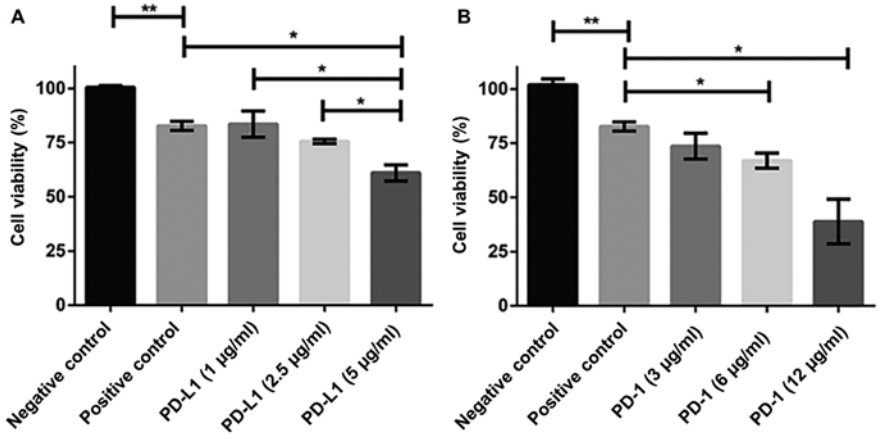

Figure 3. Cell viability in DAUDI cells treated with PD-L1 and PD-1 antibodies. Cell viability in DAUDI cells co-cultured with CIK cells and treated with (A) PD-L1 and (B) PD-1 antibodies. The negative control consisted of untreated DAUDI cells. Positive control represented DAUDI cells incubated CIK cells at a ratio of 1:1. *P<0.05; **P<0.005. PD-L1, programmed death-ligand 1; PD-1, programmed cell death protein 1; CIK, cytokine-induced killer.

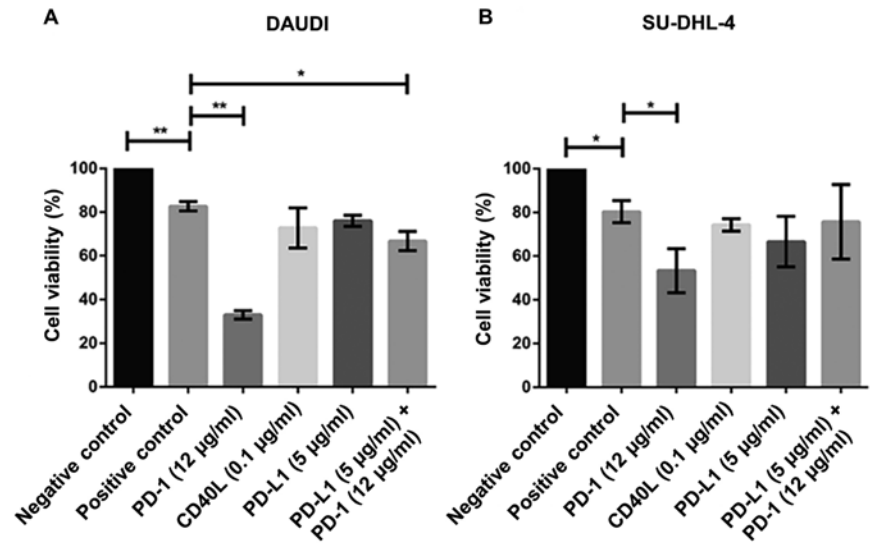

Figure 4. Cell viability in the tumor cell lines treated with antibodies prior to co-cultures. PD-1 antibody treatment in CIK cells prior to co-culture with DAUDI or SU-DHL-4 cells. Cell viability measured using the Cell Counting Kit-8 assay in (A) DAUDI and (B) SU-DHL-4 cells was evaluated when they were co-cultured with PD-1 blockade-activated CIK cells. In addition, cell viability in DAUDI and SU-DHL-4 cells was assessed when they were incubated with PD-L1 or CD40L antibodies prior to co-culture with CIK cells. *P<0.05; **P<0.005. PD-L1, programmed death-ligand 1; PD-1, programmed cell death protein 1; CIK, cytokine-induced killer.

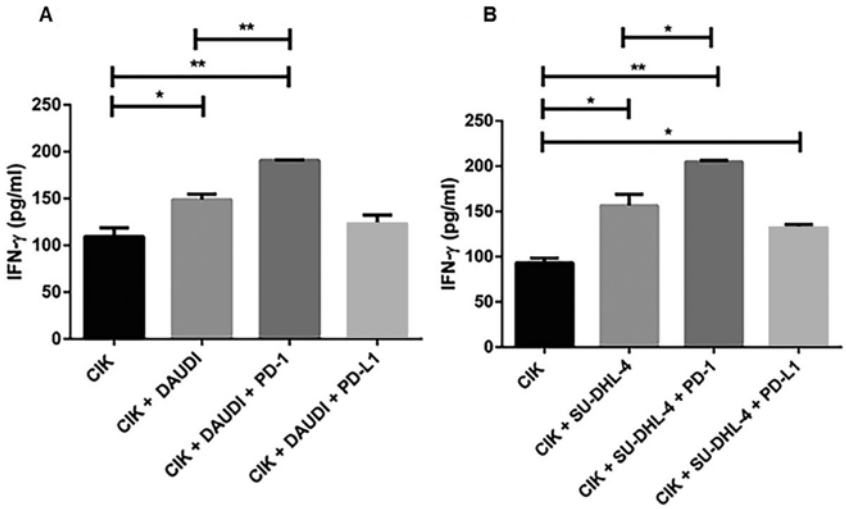

Figure 5. Secretion of IFN-γ in CIK cells co-cultured with tumor cell lines after treatment with antibodies. Secretion of IFN-γ detected by ELISA in (A) DAUDI and (B) SU-DHL-4 cells co-cultured with CIK cells and treated with PD-1 or PD-L1 antibodies. *P<0.05; **P<0.005. PD-L1, programmed death-ligand 1; PD-1, programmed cell death protein 1; CIK, cytokine-induced killer.

Discussion

Cytokine-induced killer (CIK) cell therapy has emerged as a promising option in cancer immunotherapy. There have been growing numbers of clinical trials suggesting that CIK therapy achieves a very convincing clinical response in a variety of cancers (9). In the current study, we investigated the cytotoxic capacity of CIK cells in two frequently used human B-cell non-Hodgkin lymphoma cell lines (SU-DHL-4, DAUDI). As a combinatorial approach, we also considered PD-L1/PD-1 blockade in our analyses, as it has been previously shown that combined therapy of CIK cells and PD-L1/PD-1 blockade can delay tumor growth in murine gastric cancer model (15).

Our previous study has shown that PD-1 surface expression on $CD3^{+}$ CIK cells was 3.9±0.5% (16). In our current analysis, both cell lines showed very low levels of PD-L1 and PD-L2, however, a significant increase in PD-L1 (but not PD-L2) was observed when CIK cells were co-cultured with them. To mention, the PD-L1/L2 levels may vary in other B-NHL cell lines (>100 reported in ATCC repositories), as suggested by Sharma *et al* the heterogeneity between cancer cell lines (in addition to genetic-epigenetic variations) may lead to discrepancies in the experimental data (17). In our study we could show that the variation in cell viability was entirely due to co-cultured CIK cells, thus the above-mentioned factor can be excluded. This can also be evident from the CCK-8 assay data, where we used different titrations of PD-L1 and PD-1 antibodies and obtained the exact concentrations (PD-L1: 5 μg/ml, PD-1: 12 μg/ml) required to obtain the comparable cytotoxicity levels. In contrast to PD-L1, CD40L blockade did not show any significant alteration, but it cannot be excluded that its high concentrations might exert potent cytotoxic effects.

Arguably, the question remains whether PD-1 and PD-L1 are comparable, as they are not fully interchangeable in the clinical practice. In our analysis the cell viability was severely impaired with PD-1-blocked CIK cells, in contrast to PD-L1 which showed no significant differences. However, the cumulative effect (PD-1 combined with PD-L1 blockade) was clearly seen when CIK cells were co-cultured with DAUDI cells. Importantly, we observed a significant increase in IFN-γ secretion in both cell lines when treated with CIK alone and/or with PD-1 antibody. We therefore suggest that *in vivo* experiments are warranted to undermine the extent at which PD-1 inhibitor could be used to enhance the antitumor activity of CIK cells in B-NHL.

Taken together, our *in vitro* data suggest that CIK cells can exert a significant cytotoxic function against B-NHL, and a combination of PD-1 inhibitors with CIK cells may provide a potential therapeutic option for this particular lymphoma.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

IGHSW conceived the study. YL performed flow cytometry and MWJRB contributed to the MTT, CCK-8 and ELISA assays. AS analyzed the data and revised the manuscript critically for important intellectual content. RSW and MK contributed to study design and revised the manuscript. AS, RSW, MK and

IGHSW were responsible for confirming the authenticity of the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Approval of the ethics committee of the University Hospital Bonn (Bonn, Germany) was obtained, including signed informed consent from the healthy volunteers.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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