Efficacy of cytokine-induced killer cells targeting CD40 and GITR

KAJA D. BIEDERBICK[1] and INGO G.H. SCHMIDT-WOLF[2]

[1]Department of Internal Medicine III, University Hospital Bonn; [2]Department of Integrated Oncology, CIO Bonn, University Hospital Bonn, D-53105 Bonn, Germany

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Abstract. Since the publication of a novel protocol in 1991, cytokine-induced killer (CIK) cells have shown promising results in the treatment against neoplastic diseases. Despite ongoing preclinical and clinical studies, CIK cell treatment in the context of human monoclonal antibodies targeting tumor-necrosis factor receptors remains overlooked. The present study investigated whether a combination of CIK cells with human monoclonal antibody anti-CD40 and anti-Glucocorticoid-induced TNF-related protein (GITR) would lead to further cytotoxicity against tumor cells expressing CD40 and GITR ligand (L). Therefore, *in vitro* experiments with human lymphoma cell lines SU-DHL-4 and Daudi (both CD40 positive) and human breast adenocarcinoma MCF-7 (GITRL positive) were performed and the secretion of interferon (IFN)-γ was measured. Three interesting results emerged: i) a combination of CIK cells and anti-CD40 mAb is more effective than CIK cell treatment alone; ii) the use of anti-GITR mAb and CIK cells significantly enhanced the cytotoxicity of CIK cells against MCF-7 compared with single CIK cell treatment and iii) the combination of both antibodies and CIK cells abrogates the anti tumoral effect of CIK cells on all three cell lines. By performing an ELISA for IFN-γ measurement, a lower secretion was observed when anti-CD40 or anti-GITR mAb was added. This outcome indicates that further studies *in vitro* and *in vivo* may aid in understanding the synergistic molecular mechanisms of CIK cells, and anti-CD40 and anti-GITR mAb.

Correspondence to: Professor Ingo G.H. Schmidt-Wolf, Department of Integrated Oncology, CIO Bonn, University Hospital Bonn, 25 Sigmund-Freud-Straße, D-53105 Bonn, Germany
E-mail: ingo.schmidt-wolf@ukbonn.de

Abbreviations: GITRL, Glucocorticoid-induced TNF-related protein ligand; TNF, tumor necrosis factor, NF-κB, nuclear factor-κB; IL, interleukin, MHC, major histocompatibility complex; NKG2D, natural killer group 2, member D

Key words: cytokine-induced killer cells, monoclonal antibody, anti-CD40, anti-GITR, immunotherapy

Introduction

There are certain cell surface proteins which are expressed on tumor cells and help them to suppress the immune system, grow and disperse in the human body. In this study, we tested whether this interaction could be blocked by the addition of human monoclonal antibodies targeting members of the tumor necrosis factor receptor (TNFR) superfamily. Two of these members are CD40 and glucocorticoid-induced TNF-related protein (GITR). Both of them use a cascade which requires TNFR-associated factors (TRAFs). The binding of TNFR to TRAFs initiates different signal transductions which lead among other things to the upregulation of NF-κB (1).

CD40 is normally found on antigen-presenting cells and B-cells and plays an important role in primary immune response, B-cell activation and antibody-dependent cell cytotoxity. However, it is also expressed on various hematological malignancies such as multiple myeloma (2,3), non-Hodgkin's lymphoma (4) and Hodgkin's disease (5). The ligand (L) for CD40 is also a TNF superfamily member (CD154) (6,7) and can be found on activated CD4+, CD8+ and γδ T cells (6,8). On malignant B cells, signaling via CD40 can lead to proapoptotic or antiapoptotic effects on tumor cells (9).

GITR can be found on CD4+ and CD8+ T cells as well as on natural killer (NK) cells and is highly expressed after activation (10). Studies have shown that GITRL-positive tumors such as MCF-7 use the GITR-GITRL-interaction to avoid immune response by the secretion of TNF-α, IL-6 and IL-8 (11,12).

Cytokine-induced killer (CIK) cells are a heterogenous cell group consisting of CD3+CD56+, CD3-CD56+ and CD3+CD56- T cells which exercise their cytotoxicity in a non MHC-restricted manner (13). The $CD3^{+}CD56^{+}$ subpopulation serves as the main effector cells combining T cell capability with NK cell function. Tumor lysis is finalized via NKG2D receptor and MHC-related ligands (MIC A/B) and ULBP family on tumor cells which results in up-regulated secretion of perforin and granzym (14-16). CIK cells have already been proven to be a promising treatment against malignant diseases in various preclinical and clinical studies (17-24). Recent developments in immunotherapy show interesting outcomes and our aim in this study was to find new ways to make CIK cell treatment even more effective by combining them with monoclonal antibodies which are supposed to block tumor surviving interaction between malignant cells and CIK cells.

Materials and methods

Antibodies. Human monoclonal antibody anti-GITR (clone 110416) and anti-CD40 (clone 82102) were obtained from R&D Systems Europe, Ltd., (Abingdon, UK) and dissolved in sterile phosphate buffered saline (PBS) to a concentration of 10 and 1 μg/mL for experimental use. Mouse IgG1 isotyope control and mouse IgG2B isotype control (R&D Systems) were kept in sterile PBS in a concentration of 10 and 1 μg/mL.

Tumor cells. The human breast adenocarcinoma cell line MCF-7 (DSMZ, Braunschweig, Germany) was cultured in DMEM medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (P/S) (both from Invitrogen; Thermo Fisher Scientific, Inc.) while the human lymphoma cells SU-DHL-4 and Daudi (DSMZ) were kept in RPMI-1640 medium (Pan-Biotech GmbH, Aidenbach, Germany) containing 10% FCS and 1% penicillin/streptomycin. All three cell lines were incubated at 37˚C in a humidified atmosphere of 5% CO_2.

Generation of CIK cells. CIK cells were generated as described previously (25). Peripheral mononuclear blood lymphocytes were derived from healthy donors. After having washed cells for the first time with PBS + 10% bovine serum albumin (BSA), the cell pellet was resuspended in 10 ml erythrocyte lysis puffer. Cells were then washed twice again, counted and cultured in Hepes buffered RPMI-1640 medium complemented with 10% FCS and 1% P/S at a density of 5x10^6/ml. To separate CIK cells from dendritic cells, the non-adherent cells containing medium were transferred into a new bottle after 1-2 h of incubation at 37˚C. Medium was filled up to a total volume of 40 ml and 1,000 U/ml recombinant human interferon gamma (IFN-y) (ImmunoTools GmbH, Friesoythe, Germany) was added. On day one, CIK cell received 1,000 U/ml anti-CD3 (eBioscience; Thermo Fisher Scientific, Inc.), 1,000 U/ml IL-1ß (ImmunoTools GmbH) and 300 U/ml rh IL-2 (ImmunoTools GmbH). Every third day, medium was partially changed and additional rh IL-2 (300 U/ml) was given. After 2 weeks of maturation, CIK cells were ready for experimental use.

MTT assay. In order to determine CIK cell cytotoxicity, an MTT assay was performed. Therefore, tumor cells (1x10^4 cells/well) were seeded in triplicates into round-bottomed 96 well plates. Human monoclonal antibody or isotype control and CIK cells at an effector-to-target ratio of 1:5 were added to a total volume of 100 μl/ well and the plates were incubated 24 h at 37˚C. After that, 80 μl of the supernatant was removed and 55 μl MTT-reagent 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was given to the cells. Vital cells were able to include the yellow colored reagent and reduce it to purple formazan. Cell viability depended on the amount of measured dye. After 45 min of incubation, the MTT colorant was dislodged and cells were lysed with DMSO (Carl Roth GmbH, Co., KG, Karlsruhe, Germany). The plate was shaken for ten min at 300 rpm and absorbance was measured at 560 nm.

The following formula was used to calculate cell viability: Viability (%) = 100-[(tumor cells incubated with CIK cells with or without additional antibogy -CIK only)/tumor only] x 100

This experiment was replicated at least three times with CIK cells from different donors.

Determination of cytokines. For IFN-y determination, an ELISA was done using Thermo Scientific Human IFN-y ELISA Reagent Kit (Thermo Fisher Scientific, Inc.). Tumor cells (1x10^5) were seeded in quadruplets and incubated with anti-GITR or anti-CD40 mAb (10 μg/mL) and CIK cells (E:T 1:2) in round bottomed 96-wells plates for 24 h at 37˚C. This was followed by transferring supernatant (100 μl) into 96-wells ELISA plates, precoated with IFN-y coating antibody and following the instructor's protocol IFN-y concentration, was measured at 450 nm wavelength.

Statistical analysis. For statistical analysis, GraphPad Prism v5 was used (GraphPad Software, Inc., La Jolla, CA, USA). One-way analysis of variance with Bonferroni post hoc test and Student's t-test were performed to analyze statistical significance. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effect of combining CIK cells with human monoclonal antibody anti-CD40 on lymphoma cell line SU-DHL-4 and Daudi. CIK cells only had a slightly cytolytic effect against SU-DHL-4 and Daudi when cultured alone at a very low effector-to-target ratio (1:5). By adding human monoclonal antibody anti-CD40 (1 μg/mL) the cytotoxicity of CIK cells could be increased by up to 27.19% for SU-DHL-4 (Fig. 1A) and 28.26% for Daudi (Fig. 1B). Anti-CD40 itself had no effect on the tumor cell lines. The MTT-assay was also performed using isotype control in the same concentration as anti-CD40 (1 μg/mL). No significant changes were measured (data not shown).

Effect of combining CIK cells with human monoclonal antibody anti-GITR on breast adenocarcinoma cells MCF-7. There was an improvement of cytolysis against MCF-7 when CIK cells were stimulated with anti-GITR mAb (10 μg/mL) compared to single CIK cell treatment (11.38%) (Fig. 1C). Again, we used a low E:T ratio (1:5) and for controls performed the assay under same circumstances with isotype control. No significant effect was noticed (data not shown). Co-culturing of MCF-7 and anti-GITR mAb had no effect on cell viability.

Detection of IFN-γ secretion on single CIK cell treatment and CIK cell/human monoclonal antibody treatment. To investigate further, we measured the IFN-γ secretion of CIK cells when cultured with tumor cell lines (SU-HDL-4, Daudi and MCF-7) alone (E:T 1:2) and in the presence of human monoclonal antibody anti-CD40, anti-GITR or isotype control at a concentration of 10 μg/mL (Fig. 2). After 24 h of incubation the supernatants were analyzed by ELISA. Surprisingly, the amount of IFN-γ decreases or remains the same when anti-CD40 or anti-GITR mAb is added. The use of isotype control did not lead to significant changes either (data not shown).

Combination of both antibodies anti-CD40 and anti-GITR with CIK cells on all three tumor cell lines and their effects.

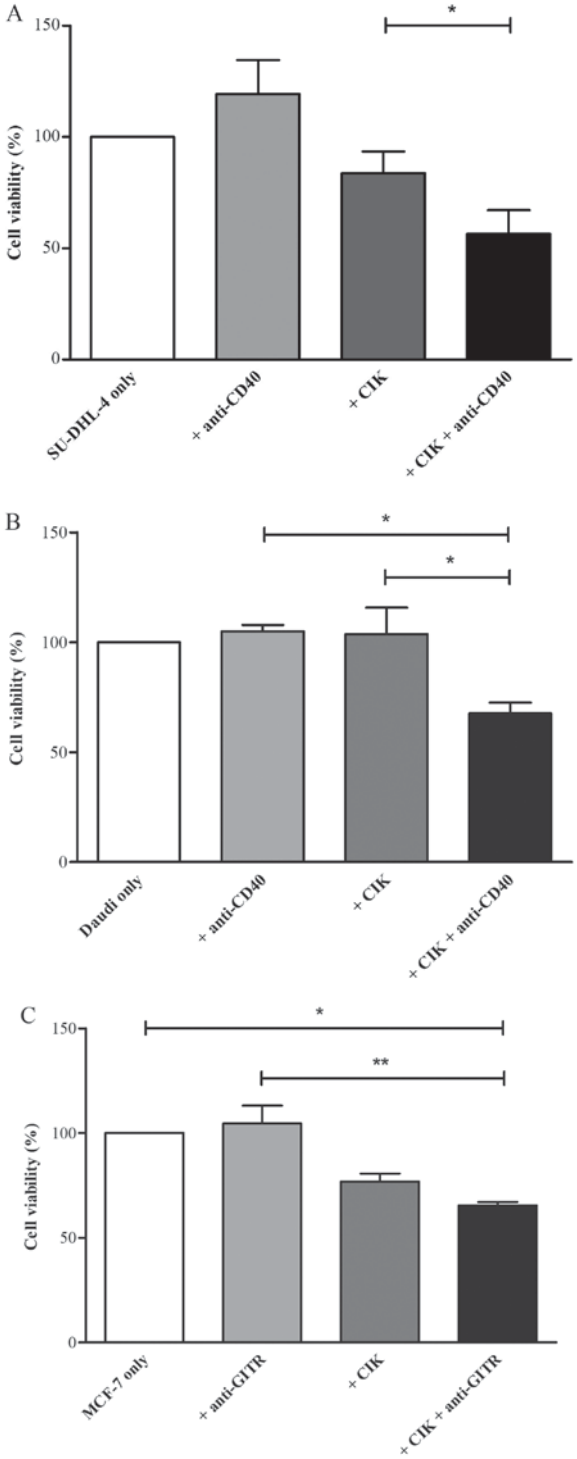

Figure 1. Cell viability was measured using MTT assay. (A) SU-DHL-4 with anti-CD40 cytotoxicity increased up to 27.19% (P=0.0346). (B) Daudi with anti-CD40 led to 28.26% higher cytotoxicity (P=0.0357). (C) MCF-7 with anti-GITR improvement of cytolysis of 11.38% (P=0.0372). Data presented as mean +/- SEM. *P<0.05 and **P<0.01. anti-GIRT, anti-Glucocorticoid-induced TNF-related protein.

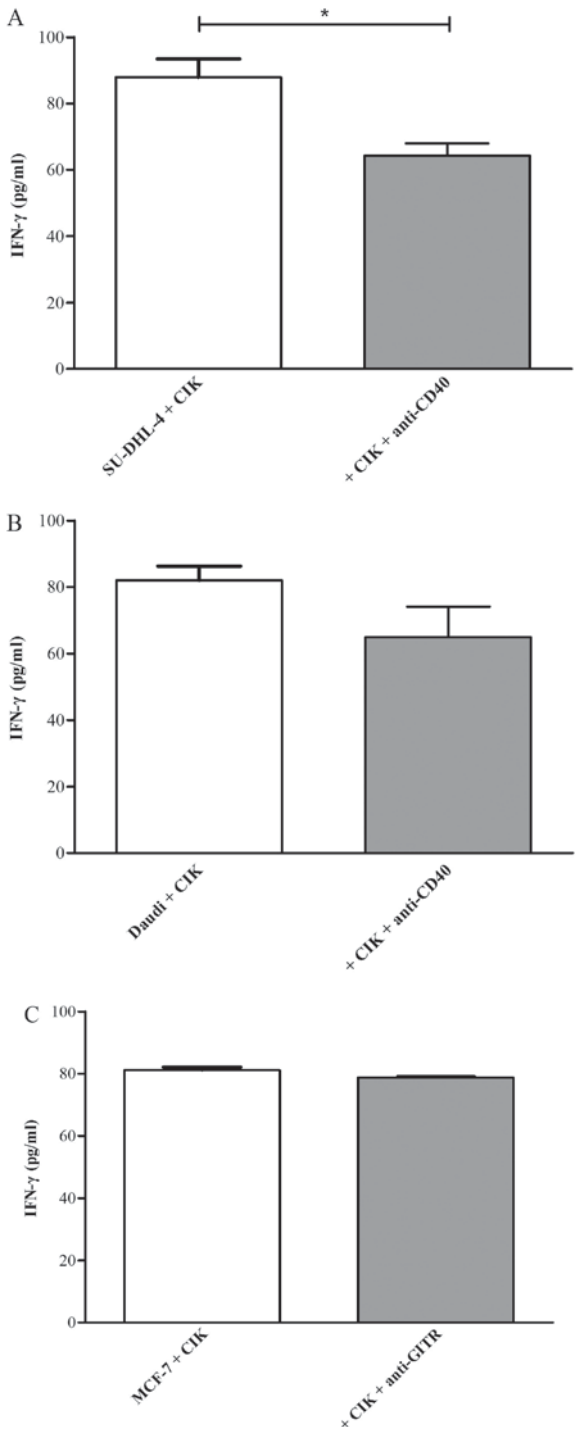

Figure 2. For IFN-γ detection, an ELISA was done with all three tumor cell lines SU-DHL-4 (A), Daudi (B) and MCF-7 (C) and CIK cells and human monoclonal antibody anti-CD40 and anti-GITR (10 μg/ml) at an effector-to-target ratio of 1:2. A lower IFN-γ secretion was measured when anti-CD40 was supplemented. For the use of anti-GITR and anti-CD40 (Daudi), no significant difference was noticed in comparison to single CIK cell treatment. *P<0.05. CIK, cytokine-induced killer; anti-GIRT, anti-Glucocorticoid-induced TNF-related protein.

We proceeded to test whether combining the two antibodies would lead to further stimulation of CIK cells. As Fig. 3 demonstrates this process has indeed, the opposite effect. By applying anti-GITR (10 μg/mL) and anti-CD40 mAb (1 μg/mL) with CIK cells on SU-DHL-4, Daudi and MCF-7, the cytolytic activity of CIK cells appeared to be decreasing on all three tumor cell lines.

Discussion

Immunomodulatory monoclonal antibodies in the treatment of malignant diseases have gained much interest over the past years. The concept is that monoclonal antibodies block the interaction between certain surface proteins on tumor cells

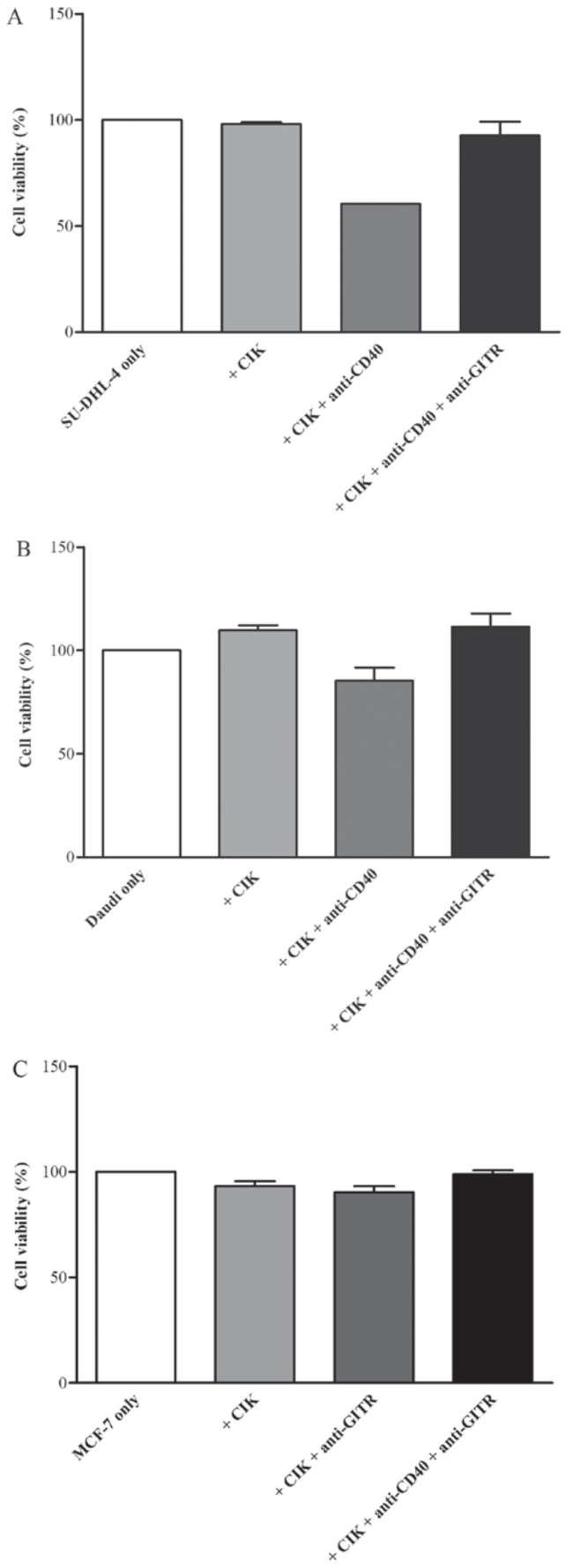

Figure 3. Tumor cell lines (A) SU-DHL-4, (B) Daudi and (C) MCF-7 were co-cultured with CIK alone, CIK cells and anti-CD40 or anti-GITR and with CIK cells and both antibodies. By applying the second antibody the cytolytic activity of CIK cells seems to be inhibited in all three samples. CIK, cytokine-induced killer.

and the immune system which normally help them to suppress immune response. Furthermore, human monoclonal antibodies might be able to induce antibody-dependent cell-mediated cytotoxicity (ADCC). It has already been reported that $CD3^+CD56^+$ cells express CD16 and therefore ADCC might be a possible mechanism on how CIK cells perform their enhanced cytolytic activity in our work (26). Since monoclonal antibodies such as rituximab and ipilimumab have already been approved as therapeutic reagents against few malignant diseases new approaches targeting other molecules than CD20 and CTLA-4 have now become the focus of study. A variety of hematological and non-hematological neoplasias express CD40, so monoclonal antibodies against CD40 might be a promising treatment option for these cell lines. Normally found on B cells, signaling via CD40 can be pro- or anti-apoptotic on B-lineage malignancies (9,27). The intracellular pathway still needs to be explored. One explanation might be the induction of *bax* after CD40-stimulation (28). As part of the *Bcl-2* family it facilitates p53 induced apoptosis (29). Humanized agonistic SGN-40 and antagonistic CHIR-12.12 have already been generated and are currently used in clinical trials (27). In our study, we showed that a combination of human monoclonal anti-CD40 with CIK cells led to increased cytotoxicity compared to CIK cell treatment alone against CD40+ lymphoma cells SU-DHL-4 and Daudi. Anti-CD40 mAb detects the corresponding surface protein with its Fab-fragment on SU-DHL-4 and Daudi while the Fc-region functions as stimulatory signal for CIK cells. Moreover, CD40 is also expressed on CD8+ T cells and following a cascade of Ras, Phosphoinositide 3-kinase (PI3K) and protein kinase C (PKC) CD40, -signaling results in down regulating T reg cells' immunosuppressive effects (30). In effect, human anti-CD40 mAb may work in one or both ways to induce anti tumor activity; the exact molecular mechanisms still remain unclear and need to be investigated further.

We tested a second combination of CIK cells with another monoclonal antibody targeting Glucocorticoid-induced TNFR (GITR). Here, the cytolytic activity of CIK cells seems to be enhanced by stimulation with human anti-GITR mAb. GITR is found on CD4+-, CD8+-, NK- and Treg cells where as its ligand, GITRL, is constitutively expressed on tumor cell lines like MCF-7 and uses the GITR-GITRL-interaction for immunosurveillance. The Salih group reported that by GITR-stimulation the NF-κB activity in NK cells was decreased and could be partially regained after addition of anti-GITR. This data indicates that GITR-induced reduction of NF-κB may explain how GITRL-expressing tumors escape immune defense (10).

Since we examined a higher cytolytic activity of CIK cells by the addition of anti-CD40 or anti-GITR mAb, we expected a correlating increase in IFN-γ production when CIK cells were stimulated with human monoclonal antibodies. However, we found that the opposite was true. With human lymphoma cells SU-DHL-4 and Daudi, a lower secretion of IFN-γ was measured and no changeable amounts with MCF-7. Partially, these -γ increase when anti-GITR was added could not be explained.

Finally, we tested the cytolytic activity of CIK cells when anti-CD40 and anti-GITR mAb were incubated with all three cell lines. In each sample CIK cells' cytotoxicity was inhibited.

This data has led us to the conclusion that CD40 and GITR share a common pathway. Both molecules belong to the TNFR superfamily and use TRAF proteins for signal transduction (31-33).

Our results confirm the works of Baltz *et al* Only the treatment with IL-15 enhanced NK cells' production of IFN-γ while untreated NK cells showed minor or no effect (10). Why IL-15 was necessary for IFN. This might be an explanation why the simultaneous use of anti-GITR ant anti-CD40 mAb lowered CIK cells' cytotoxicity in comparison to CIK cell treatment with one monoclonal antibody. To sum up, the combination of CIK cells and human monoclonal antibodies showed promising results *in vitro*. Our findings confirm the works of Cappuzzello *et al* who recently reported that CIK cells are capable of ADCC and their cytolytic activity increased when monoclonal antibody was added (26). This opens up a variety of combinations between CIK cells and different monoclonal antibodies such as anti-CD137, anti-CD134 (OX40), anti-CD152 (CTLA-4), anti-PD-1 and anti-PD-L1. Most of them are under current clinical investigation (34). Anti-CD137 and anti-CD30 have already been tested with CIK cells (35,36). Immunotherapy, especially the use of monoclonal antibodies, has the potential to replace the present treatments against cancer since it is a restricted therapy targeted to tumor and carries less side effects, and as such, efforts in this field must continue.

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Availability of data and materials

All data used and analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

KB performed the experiments, analyzed the data and wrote the essay. ISW designed and supervised the study, and corrected the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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