A CTLA-4 Blocking Strategy Based on Nanobaby in Dendritic Cell-stimulated
Cytokine-induced Killer Cells Enhances Their Anti-tumor Effects

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Abstract

Background: Cytokine-induced killer cells which were induced with tumor antigen-pulsed dendritic cells (DC-CIK) immunotherapy is a promising strategy for the treatment of malignant tumors. However, the efficacy was restricted by the immunosuppression of tumor microenvironment mediated by the cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) pathway. We, therefore, screened a nanobody which is targeted for CTLA-4 (Nb36), and blocked the CTLA-4 signaling with Nb36 to overcome the negative co-stimulation of effector T cells.

Methods: Peripheral blood mononuclear cells (PBMCs) were collected from healthy donors and used to induce CIK cells in vitro, then co-cultured with DC cells that have received tumor antigens. We tested whether blocking CTLA-4 signaling with Nb36 could promote DC-CIK cells proliferation, pro-inflammatory cytokine production and cytotoxicity in vitro. In vivo experiments, The NOD/SCID mice were injected subcutaneously with HepG2 cells to induce solid tumor. We observe whether this therapy can more effectively inhibit tumor growth in mice.

Results: After being stimulated with Nb36, DC-CIK cells presented enhanced proliferation and production of IFN-γ in vitro, thereby strengthening the killing effect on tumor cells. For in vivo experiments, Nb36-treated DC-CIK cells significantly inhibited the growth of subcutaneously transplanted tumors of liver cancer, reduced

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tumor weight and prolonged the survival of tumor-bearing NOD/SCID mice.

**Conclusions:** These findings demonstrated that in response to CTLA-4 specific nanobody stimulation, DC-CIK cells exhibited superior anti-tumor efficacy. Our findings suggested that DC-CIK cells + Nb36 has potential to treat malignant tumors through in vivo adoptive therapy.

**Keywords:** nanobody, cytotoxic T-lymphocyte antigen-4, Cytokine-induced killer cells

**Background**

Cancer immunotherapy exerts beneficial effects through mediating tumor cell regression, which relies on the activation, persistence and targeting of anti-tumor T cells[1]. Cytokine induced killer cells (CIK) are immunologically active cells that are expanded in vitro, which have both the strong cytotoxicity of T lymphocytes and the non-mhc-restricted killing characteristics of NK cells[2]. Dendritic cells (DCs), among the most powerful antigen-presenting cells, can capture the antigen and present to the surface of a responder (such as CIK), thereby activating the antigen-specific immune response and improving the function of effector cells. DC-CIK therapy has the potential for both MHC-unrestricted and tumor-specific activity[3]. However, it has been identified that the cytotoxicity and proliferation of DC-CIKs in vivo are restricted by the immunosuppression of tumor microenvironment in the solid tumor therapy[4]. In addition to dysfunctional stromal cells in the tumor microenvironment, there are various regulatory T cells, myeloid-derived suppressor cells, and up-regulated tumor suppressor molecules such as cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) and programmed cell death protein-1 (PD-1)[5-6]. The complex tumor microenvironment has become a hotbed for tumor development, leading to the exhaustion of infused DC-CIK cells, which is the main mechanism that limits the efficacy of DC-CIK adoptive therapy[7].

CTLA-4 is a protein receptor mainly expressed in activated T cells and regulatory T cells. CTLA-4 competes with CD28 for binding to ligands and emits inhibitory
signals to attenuate T cell activation[8]. Expression of CTLA-4 is usually up-regulated with the continuous activation of T cells. CTLA-4 can be combined with molecules of B7 family on the surface of DCs as a co-stimulatory signal to inhibit the proliferation, activation and cell cycle of T cells, leading to decreased secretion of cytokines such as IL-2, IL-4, IFN-γ and expression of IL-2 receptors. This makes tumors escape immune surveillance by negatively regulating the proliferation of T cells[8-10]. As one of the most important immunosuppressive receptors, CTLA-4-targeted immune checkpoint inhibitors are also a hot topic in tumor immunotherapy. The blockade of the CTLA-4 signaling with monoclonal antibodies leads to enhanced antitumor immune response, which becomes a potential tumor immunotherapy strategy and is undergoing a number of clinical investigations[11-12]. However, this strategy has many limitations, including non-specific binding of monoclonal antibodies to normal tissue and heterogeneous tumor antigens, and poor penetration of antibodies in tumor microenvironment. Therefore, it is urgent to develop novel antibodies with high efficiency and low toxicity[13].

Nanobody (Nb) is a special single domain antibody which is derived from camelids. A special single-chain antibody (lacking light chain and CH1) naturally exists in this animal. Nb is obtained after cloning its variable regions, which is the smallest antigen-binding unit ever found[14]. Simple molecular structure allows Nb to bind to epitopes that are not easily accessible to traditional antibodies[15]. In addition, Nb has a strong tolerance to changes in temperature and pH, and its stable conformation allows it to be taken orally. Compared with conventional antibodies, Nb has high specificity, good physical and chemical stability, high yield, low cost, and lacks immunogenicity, making it suitable in the clinical setting [16]. In previous studies, we obtained a set of CTLA-4 specific Nb using the phage display technology. This anti-CTLA-4 Nb (Nb36) can bind to CTLA-4 epitopes on the surface of activated T cells in vitro[17-18]. To further investigate its antitumor activity, in the present study, we used the novel antibodies to block the CTLA-4 signaling in activated DC-CIK cells, thereby promoting DC-CIK to survive, proliferate and infiltrate(Figure 1).
Materials and methods

Animals and Cell Culture
Female NOD/SCID mice, aged 4-6 weeks, were purchased from Beijing Vital River Lab Animal Technology (Beijing, China) and raised in the SPF environment. All protocols were approved by the Animal Ethics Committee of Hainan Medical University. HepG2 (hepatoma carcinoma) and A549 cells (lung carcinoma) were purchased from the International Nanobody Research Center of Guangxi and cultured in DMEM supplied with 10% fetal bovine serum (FBS) and penicillin/streptomycin in 37 °C, 5% CO₂.

Antibodies and Nanobodies
The anti-CTLA-4 Nb (Nb36) and anti-CD105 Nb were developed in our laboratory. Anti-CTLA-4 mAb, anti-CD3 mAb, anti-CD56 mAb, anti-CD80 mAb, anti-CD83 mAb and anti-MHC II mAb were purchased from Abcam (Cambridge, UK).

Generation of CIK and DCs
Peripheral blood mononuclear cells (PBMCs) were collected from healthy donors by density gradient centrifugation, and cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco) containing 10% FBS and penicillin/streptomycin. Following 4 h of culture, the suspended cells (T cells) were grown to generate CIK cells in RPMI-1640 with 10% FBS containing 500 ng/mL anti-CD3 antibody, 100 U/mL IFN-γ (Servicebio, Wuhan, China) and 10 μg/mL polyhydroxyalkanoates (Solarbio, Beijing, China). In addition, adhered cells were used for dendritic cell differentiation via culturing in RPMI supplemented with 1000 U/mL recombinant human GM-CSF (rhGM-CSF; R&D, MN, US) and 500 U/mL rhIL-4 (R&D, MN, US) for 7 days. Next, A549 and HepG2 cells were lysed (Repeated freeze-thaw procedure), and the supernatant was obtained as the tumor antigen. On day 8, the supernatant and 10 ng/mL TNF-α and 10 ng/mL IL-1β were added to the DCs medium, after which the culture was maintained for two more days. Then, DCs and CIKs were co-cultured at a
ratio of 1:10 for two days. On day 12, we added the Nb36 to DC-CIK cells to mediate CTLA-4 blockade. The study were approved by the local ethics committee of Hainan Medical University.

**Phenotypic Analysis of CIK and DCs**

On day 7, the HLA-DR, CD80 and CD83 expression of DCs were analyzed by flow cytometry. Then, phenotypic analysis of DC-CIK cells, including CD56 and CD3 was performed by flow cytometry on day 14.

**Cell Proliferation Assay**

Proliferation of DC-CIK cells was determined by CFSE staining in vitro. Individual groups of DC-CIK cells were stimulated with Nb36 (50 μg/mL), anti-CTLA-4 mAb (50 μg/mL), and anti-CD105 Nb (Isotype Control, 50 μg/mL) (1 × 10^6 cells/tube), and then labeled with CFSE (Sigma-Aldrich, MO, US) at 37 °C for 5 min. After washing, the cells were stimulated with the same number of HepG2 cells that had been irradiated (100 Gy) for 120 h. The suspended T cells were harvested and the percentage of proliferative T cells was determined by flow cytometry.

**Cytotoxicity Assay**

We next assessed the cytotoxicity of DC-CIK cells against HepG2 and A549 cells in vitro. After being stimulated with Nb36, anti-CTLA-4 mAb, or anti-CD105 Nb, DC-CIK cells were collected and mixed with HepG2 or A549 cells with E:T ratio of 5:1, 10:1 or 20:1. Target cells with PKH26 staining were co-cultured for 6 h at 37 °C, 5% CO₂. The PKH26-labeled cells were stained with propidium iodide (PI) and the percentage of PKH26‘PI’ dead cells was analyzed by flow cytometry.

**ELISA and ELISPOT Assays**

DC-CIK cells and T cells (control, 1 × 10^5/well) were co-cultured with HepG2 cells (1 × 10^5/well) for 16 h. The levels of TNF-α, IL-2, and IL-10 in the supernatants were determined by ELISA (BD, NJ, US) according to the manufacturer's protocols. To test
the density of IFN-γ secreting cells, ELISPOT assay was performed. Briefly, DC-CIK cells and T cells (control, $3 \times 10^5$/well) were stimulated in triplicate with irradiated HepG2 cells ($1 \times 10^5$/well) at 37 C overnight in 96-well plates that had been coated with anti-IFN-γ. After washing, the captured IFN-γ was reacted with biotinylated anti-IFN-γ at 4 °C overnight. Subsequently, the IFN-γ specific immunocomplex was detected with streptavidin-AP and visualized with a substrate solution (BCIP/NBT). The numbers of spot forming cells in the wells were confirmed on a CTL ImmunoSpot S6 Ultimate-V analyzer with Immunospot software, version 5.1 (OH, US).

**Xenograft Experiments in Mice**

For xenograft experiments, each NOD/SCID mice was implanted with HepG2 cells ($2 \times 10^6$/injection) via subcutaneous injection in the left armp. Once the tumors reached a volume of 100 mm$^3$, tumor-bearing mice received $5 \times 10^6$ DC-CIK cells, Nb36-treated DC-CIK cells, anti-CTLA-4 mAb-treated DC-CIK cells, anti-CD105 Nb-treated DC-CIK cells, or PBS every seven days for three times. The volume of tumors was recorded with a vernier caliper every three days to evaluate tumor growth. Tumor volume (V) was calculated according to the formula: $V = 0.5ab^2$, where a is the largest diameter, and b is the perpendicular diameter.

**Immunohistochemistry**

The tumor tissue samples were fixed with 10% neutral formalin before paraffin embedding, and the slice thickness was 4-µm. After that, these sections were incubated with anti-ki67 monoclonal antibody overnight at 4 °C. After washing twice, sections were further incubated with the HRP-labeled secondary antibody. Images were obtained with a microscope (Nikon, Japan).

**Statistical Analysis**

The FACS data was analyzed using FlowJo 7.6 software. The Statistical analysis of data was performed by GraphPad Prism software 6.0. The differences among different
groups in the in vitro and vivo experiments was performed to evaluated using One-way ANOVA with Tukey’s multiple comparison. The tumor growth curve was analyzed with two-way ANOVA with correction for Tukey’s multiple comparison. The survival curve of animals in the difference between groups was calculated by the Kaplan-Meier analysis (log-rank test).

**Results**

**Characterization of the Nb36-treated DC-CIK cells**

Flow cytometry was used to monitor the immunophenotype of the ex vivo cultivated cells. The high expression of HLA-DR (89.4±3.55%), co-stimulatory molecules CD80 (99.2±0.7 %), and the maturation marker CD83 (47.3±2.38 %) on day 7 demonstrated the maturation of DCs (Figure 2A). These DCs were stimulated by tumor antigens and incubated with CIK. The CIK cells had the CD3^+CD56^+ phenotype in the DC-CIK+Nb36 group with a median percentage of total cells of 46.43% (range 43.1%-48.4%), which was significantly higher than the untreated DC-CIK group (33.46%, range 32.1%-36.1%) and close to the CTLA-4 mAb treated group (46.16%, range 42.8%-49.42%) (Figure 2B). These results indicated more efficient activation and expansion of DC-CIK cells when the CTLA-4 signaling was blocked.

**Blocking CTLA-4 signaling with Nb36 promoted proliferation of DC-CIK Cells in vitro**

We tested whether blocking CTLA-4 signaling with anti-CTLA-4 Nb could promote DC-CIK cells proliferation and pro-inflammatory cytokine production in vitro. Our results showed that stimulating with tumor cells (HepG2) for 120 h significantly promoted the proliferation of CFSE-labeled DC-CIK cells in the DC-CIK+Nb36 group than in the DC-CIK (p<0.001) and DC-CIK+CTLA-4 mAb (p<0.05) group (Figure 3A-B). Longitudinal measurements of Nb36-treated DC-CIK cells proliferation suggested that the number of CFSE-labeled DC-CIK cells was significantly greater than in other groups except the DC-CIK+CTLA-4 mAb group. After 11 days of cultivation, the number of DC-CIK cells in the DC-CIK+Nb36 group
increased by 180 times (Figure 3C). These data indicated that blocking CTLA-4 signaling with Nb36 further activated DC-CIK cells and promoted cell proliferation in vitro.

**Blocking CTLA-4 signaling with Nb36 facilitated the tumor cell killing of DC-CIK cells in vitro**

A cytotoxicity assay was performed to examine the impact of Nb36-treated DC-CIK cells on target cell killing. The results indicated that the effector-target ratios of (E:T) 5:1, 10:1 or 20:1, effector cells in the DC-CIK+Nb36 group exhibited much higher killing effects on HepG2 and A549 cells, while the T cell, DC-CIK and DC-CIK+CD105 Nb cells had low or little cytotoxicity against such target cells (Figure 3D-E). Hence, cells in the DC-CIK+Nb36 group effectively and specifically killed target cells in vitro, and the efficiency was close to the DC-CIK+CTLA-4 mAb group.

**Blocking CTLA-4 signaling with Nb36 stimulated DC-CIK Cells activation and pro-inflammatory cytokine production in vitro**

We tested whether blocking CTLA-4 signaling with Nb36 could stimulate DC-CIK Cells activation and pro-inflammatory cytokine production in vitro. Groups of DC-CIK cells were stimulated with the same number of HepG2 cells for 12 h. Then, levels of IL-2, TNF-α and IL-10 in the supernatants were measured by ELISA. We found that the levels of IL-2 and TNF-α in the supernatants of cultured Nb36-treated DC-CIK group were significantly higher than in the DC-CIK and anti-CD105 Nb-treated DC-CIK group after being challenged with target cells (Figure 4A). However, there was no significant difference in the expression of IL-10 among the different groups (Figure 4A). ELISPOT revealed that the number of IFN-γ-secreting spot forming cells in the Nb36-treated DC-CIK group was significantly greater than in the T cells, DC-CIK cells and DC-CIK+CD105 Nb groups after being challenged with HepG2 cells (Figure 4B). These data demonstrated that Nb36-treated DC-CIK cells have activated T cells and secreted higher levels of pro-inflammatory cytokines after
being challenged with target cells.

**Nb36 increased the anti-tumor efficacy of DC-CIK cells in vivo**

We examined the effects of Nb36-treated DC-CIK Cells in vivo. Treatment with DC-CIK Cells+Nb36 significantly reduced the volume and weight of tumor in mice, and prolonged the survival of tumor-bearing mice compared with PBS, T cells, DC-CIK and DC-CIK+CD105 Nb groups (Figure 5A-B). Similarly, the immunohistochemical analysis of the xenograft tumor tissue revealed that the numbers of anti-Ki67 cells in the mice receiving Nb36-treated DC-CIK cells were significantly less than other groups (Figure 5C). These data indicated that treatment of with Nb36-treated DC-CIK cells preferably inhibited the growth of implanted tumors in mice.

**Discussion**

Compared with traditional methods, tumor immunotherapy has become a promising strategy for the treatment of malignant tumors. Numerous clinical studies have shown that the activation of anti-tumor effector cells for adoptive introduction into the patient can improve the prognosis[19-20]. The sub-group of CD3⁺CD8⁺cytotoxic T cells induced by DCs is important in specific cellular immune responses. DC-CIK cells have the potential for both MHC-unrestricted and tumor-specific cytotoxicity after adoption[21]. The induction with anti-CTLA-4 monoclonal antibody promoted DC-CIK cell proliferation and differentiation into CD3⁺CD56⁺ NK T cells, which is the main effector of DC-CIK cells as it provides synergistic antitumor effects by up-regulating the secretion of pro-inflammatory cytokine and decreasing the production of immunosuppressive cytokines in vivo[22].

The CTLA-4 pathway delivers inhibitory signals that negatively regulate effector T cells to inhibit their activation. Several studies have revealed that the effects of DC-CIK cells were combined with monoclonal antibodies targeting CTLA-4[23]. However, the large molecular weight of monoclonal antibody drugs limits their
penetration and concentration in solid tumors, resulting in poor effectiveness in the tumor treatment. Moreover, the high cost prevents its wide use[24]. To overcome the above disadvantages, novel antibodies including single chain antibodies, genetically-engineered antibodies and nanobody have been developed[25]. Given the unique structural and molecular characteristics of Nb that allows them to penetrate into the tumor tissues easily[26], Nb-targeting CTLA-4 could be an effective strategy to enhance the effect of DC-CIK cells.

Our study successfully obtained CIK cells from PBMCs, as was shown after expansion in vitro for 12 days, by sequentially adding anti-CD3 antibody, IFN-γ, and PHA. Previous studies showed that CIK cells have dual properties of NK and T cells[27], which is consistent with our research by positive expression of immune markers of CD3 and CD56. DCs are the most significant antigen-presenting cells that stimulate effector T cells to enhance the immune responses. We lysed the whole tumor cells to generate tumor antigens for inducing DC maturation. This method stimulates immunity against the whole tumor antigens that induces a more complete cytotoxicity than stimulating with specific tumor antigens[28]. We found that after DC co-cultivation, the proliferation activity of CIK cells was enhanced, which was more pronounced as the CTLA-4 was blocked. The levels of pro-inflammatory cytokines viz TNF-α and IL-2 except IL-10 in the culture medium of DC-CIK+Nb36 group were all increased following co-culture with target cells, compared to the control group. The in vivo treatment of Nb36-treated DC-CIK cells displayed inhibited tumor growth and prolonged survival of human tumor xenograft mice. Immunohistochemical analysis displayed a decreased number of cells with proliferative marker Ki67. Accordingly, our study showed that Nb36-treated DC-CIK cells effectively killed target cells in vitro and in vivo. By binding to CTLA-4 on the surface of effector lymphocytes, Nb36 increased DC-CIK cells activation, with stronger cytotoxicity and anti-tumor effects.

**Conclusions**
This was the first study to report the application of nanobodies as CTLA-4 blockers to enhance the efficacy of DC-CIK cells. This preclinical study presented a new strategy and a promising prospect for the immunotherapy of malignant tumors. The results encouraged further research on the efficacy of patient-derived DC-CIK cells combined with anti CTLA-4 nanobody in the treatment of malignant tumors.

**Declaration**

**Abbreviations**

Nb: Nanobody; DC-CIK: cytokine-induced killer cells which were induced with tumor antigen-pulsed dendritic cells; CTLA-4: cytotoxic T lymphocyte-associated antigen-4; PBMC: peripheral blood mononuclear cells.

**Consent for publication**

Not applicable.

**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Ethics approval and consent to participate**

Ethical approval was obtained for collection of human PBMC samples from the local ethics committee of Hainan Medical University and in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Written informed consent was signed all volunteer. All procedures involving animal care and use were approved by the Animal Ethics Committee of Hainan Medical University and were in accordance with the National Policy and Regulations on Use of Laboratory Animals. All animal experiments were carried out in compliance with
the ARRIVE guidelines.

**Competing interests**
The authors declare that they have no competing interests.

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**Authors’ contributions**
AL, XL and WW conceived and designed the present study. WY, NH, XL and YP performed the molecular and cellular experiments in vitro; KZ and XW performed in the analysis of in vivo mouse model. WW managed the written of the manuscript.

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Figure legends

Figure 1. Strategy of therapy with DC-CIK cells+ anti-CTLA-4 nanobody. DC-CIK cells were generated. Then, nanobody against CTLA-4 (Nb36) eliminated immunosuppression via blocking CTLA-4-mediated negative co-stimulation in effector DC-CIK cells. Finally, DC-CIK cells were transferred to kill tumor cells.

Figure 2. Main phenotypes of DC-CIK cells derived from donors. (A). DCs exhibited the expression of HLA-DR and co-stimulatory molecules (CD83 and CD80) on day 7 in FCs. (B). DC-CIK cells in different groups exhibited CD3/CD56 expression in FCs. n=3, **P < 0.01, *** P < 0.001.

Figure 3. Nb36 promoted DC-CIK cells proliferation and eliminated target cells. (A). CFSE-labeled DC-CIK cells were mixed with different antibodies and co-cultured for 120 h. Proliferation of DC-CIK cells was assessed via flow cytometry. (B). Quantitative analysis of proliferation frequency of DC-CIK cells. n=3, *P < 0.05, *** P < 0.001. (C). The expansion folds of CAR T cells co-cultured with HepG2 or HepG2-FAP. n=3. (D and E). The DC-CIK cells were co-incubated with the indicated cells labeled with PKH26 at E/T ratios of 5:1, 10:1 and 20:1 for 6 h and the ratios of PHK26+PI+ were measured by flow cytometry. Nb36-treated DC-CIK cells had enhanced cytotoxicity against HepG2 and A549 target cells. n=3.

Figure 4. Nb36 increased the abundance of IFN-γ-secreting effector DC-CIK cells. (A). Nb36-treated DC-CIK cells produced higher levels of pro-inflammatory cytokines viz TNF-α and IL-2 except IL-10. DC-CIK cells were incubated with tumor cells for 16 h, and the levels of pro-inflammatory factors in the supernatants were determined by ELISA. (B). ELISPOT analysis of the frequency of IFN-γ-secreted
DC-CIK cells. \( n=3, \quad *** P < 0.001 \).

Figure 5. In vivo antitumor activities of Nb36-treated DC-CIK cells in the established subcutaneous human tumor xenografts. (A) The tumor growth curves. Nb36-treated DC-CIK cells significantly reduced the volumes of tumors. \( n=6, \quad *** P < 0.001 \). (B) The survival of tumor-bearing mice. Nb36-treated DC-CIK cells prolonged the survival of tumor-bearing mice. \( n=6, \quad *** P < 0.001 \). (C) Quantitative analysis of the CD105 expression. Data are representative images (magnification \( \times 400 \)) or expressed as the mean \( \pm \) SD from 5 randomly selected fields of tumor thin sections. \( n=6, \quad *** P < 0.001 \).