



# Anti-tumor activity of dendritic cell-cytokine induced killer cells (DC-CIKs) sensitized to HER2 against HER-positive breast cancer cells

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**ABSTRACT.** This study aimed to investigate the cytotoxicity of cytokine-induced killer cells (CIKs) and Her2 epitope peptide-sensitized dendritic cells (DCs), when co-cultured with Her2-positive MCF-7 cells. DCs were separated from the Her epitope peptide-sensitized peripheral blood; the Her epitope combines directly with the MHC-II molecule on the DC surface. The DCs were co-cultured with autologous CIKs. Lactate dehydrogenase (LDH) and ELISA kits were used to detect cytotoxicity of CIKs against MCF-7 breast cancer cells; IL-12 and IFN- $\gamma$  levels were also analyzed in the supernatant of the culture medium. CIKs activated by DCs sensitized by anchored Her polypeptide antigen have greater cytotoxicity against MCF-7 than CIKs alone or non-anchored antigen sensitized DCs-CIKs ( $P < 0.01$ ); the IL-12 and IFN- $\gamma$  levels in the supernatant were higher than that of the control ( $P < 0.01$ ). In conclusion, DCs anchored by polypeptide antigen alone or in combination with effector cells can be used to develop therapeutic DC vaccines against breast cancer.

**Key words:** Her2; Dendritic cells; Cytokine-induced killer cells; AE37

## INTRODUCTION

Similar to other middle and low-income countries, breast cancer prevalence in China is on the rise. The median age of onset in Chinese women is lower than in Western white women, owing to differences in genetic and related risk factors. Although the risk of breast cancer in Chinese women is lower than that of women in developed countries, younger Chinese women are at higher risk than their previous generations. Most recently, the annual breast cancer incidence and related deaths in China account for 12.2% and 9.6% of worldwide estimates, respectively (Fan et al., 2014). The current therapies for breast cancer include surgery, chemotherapy, radiotherapy, and immunotherapy. However, most of these are high risk or invasive procedures. Additionally, some cancers such as in women expressing the HER-2/neu protein (Pritchard et al., 2006) have a high recurrence rate. HER-2/neu is a member of the epidermal growth factor receptor family, and is normally expressed at fetal development stages. It is also found to be overexpressed in 30% of all breast cancer cases, making it a prominent target of various drug and immune treatments (Bailur et al., 2015; Martin et al., 2015). The immunogenicity peptide of HER-2/neu stimulates, recognizes, and kills the cytotoxic T lymphocyte (CTL) of tumor cells expressing HER-2/neu *in vitro* (Martin et al., 2015). Some polypeptides like E75 and GP2 are used as clinical vaccines in breast cancer patients who are HER-2/neu positive (Peoples et al., 2005; Mittendorf et al., 2006), as they can safely and effectively stimulate an antigen-specific immune response. More importantly, they can also prevent the repeated occurrence of breast cancer (Peoples et al., 2008). However, the curative effects of these vaccines are limited (Peoples et al., 2008). Therefore, it might be necessary to induce and establish the long-term immunity using a CD4+T auxiliary polypeptide (Hirschhorn-Cymerman et al., 2012; Tussiwand et al., 2012). As indicated by phase I clinical trials, AE37, a polypeptide inducing CD4+T cells, can be used to treat breast or prostate cancer patients in whom HER-2/neu protein is overexpressed (Holmes et al., 2008; Perez et al., 2010).

Dendritic cells (DCs) are essential in maintaining innate and acquired immunity. In 2010, the FDA approved APC8015 (Provenge), a vaccine for refractory prostate cancer, indicating that other effective DC vaccines may have favorable prospects. DCs can be also used with effector cells like NK cells, CTL cells, CIKs, and gdT cells for treating tumors (Cheng and Li, 2015). The *in vitro* antigen loading of DCs usually occurs through pyrolysis. RNA, DNA, viral proteins, and protein antigen of tumor cells, can sensitize DCs; the epitope peptide and MHC molecules need to form complexes. These antigen-sensitized DCs can be used as a vaccine or to elicit an immune response by effector cells. In this study, anchoring antigen AE37 was directly used to load DCs to explore their cytotoxicity against cytokine killer cells and MCF-7 cells overexpressing HER-2/neu, *in vitro*.

## MATERIAL AND METHODS

### Polypeptide antigen

li-Key/ HER-2/neu MHC MHC II polypeptide AE37 (Ac-LRMKGVGSPYVSRLLGICL-NH<sub>2</sub>), AE36(GVGSPYVSRLLGICL) were synthesized by Qiangyao Biological Technology Co., Ltd, Shanghai, China; high performance liquid chromatography (HPLC) proved > 95% purity. AE37 was dissolved with methyl-sulfoxide at a concentration of 10 mg/mL and stored at -20°C, until use. FITC-marked AE37, Cy5-marked AE37, and polypeptide-unrelated R187 (HBs aa183-191, FLLTRILTI) were synthesized and marked by Qiangyao Biological Technology Co., Ltd, Shanghai, China.

## DC culture

PBMCs were separated from the peripheral blood of healthy people recruited at the medical center. This study was conducted in accordance with the Declaration of Helsinki; the study was also approved by the Ethics Committee of Henan provincial People's Hospital. Written informed consent was obtained from all participants. PBMCs were cultured with RPMI1640 complete medium containing 10% FCS in the incubator at 37°C, in 5% CO<sub>2</sub> for 4h. After being rinsed with RPMI 1640 several times, the cells were cultured in complete medium containing rhGM-CSF (20 ng/mL, PeproTech) and rhIL-4 (20 ng/mL, PeproTech). On Day 2 and 4, the supernatant was removed and replaced by fresh culture medium containing rhGM-CSF and rhIL-4. After 48 h, the granular leukocytes that had not adhered were removed. The DC phenotypes such as CD40, CD80, and CD86 were analyzed by flow cytometry.

## DC sensitization

At Day 3, 1 mg/mL FITC-AE37 and Cy5-AE37 were added to the DC culture, and 24 h later the results were observed. Specifically, Cy5-AE36 was added first, and 12 h later, FITC-AE37 was added. Another 12 h later, the results were observed under a fluorescence microscope (Olympus AX80, Olympus, Tokyo, Japan).

## CIK culture and determination of cell surface markers

PBMCs at a density of 5x10<sup>6</sup>/mL were seeded into a 75 cm<sup>2</sup> cell culture flask, and 10 µL IL-1 $\alpha$ , IFN- $\gamma$ , and rat-anti CD3 were added per flask. After 24 h, 10 µL IL-2 was added per flask and the culture was continued. Every two days, equal volumes of culture medium was added. After 24-48 h of addition of stimulating factors to CIKs, small clonal groups were seen, reaching peak numbers at 72 h. If we observed crowding of clonal groups, they were dispersed with a dropper and more medium was added. Cell growth was observed twice daily, and the medium was supplemented according to the growth of clonal groups and medium color. During the entire process, we only added fresh media and the old media was never discarded. As the cells proliferated, they were passaged and seeded into more flasks. The cells were seeded at a density of 5 × 10<sup>6</sup>/mL into 75 cm<sup>2</sup> cell culture flasks and 10 µL IL-1 $\alpha$ , IFN- $\gamma$ , and rat-anti CD3 antibodies were added to the CIKs. After 24 h, 10 µL IL-2 was added and the culture was continued. Every two days, equal volume of culture medium was added to the flasks. On Day 10, CD3-PE, CD56-FITC, CD4-FITC, and CD8-FITC antibodies were used to detect surface markers by flow cytometry.

## DC-CIK co-culture

On Day 5, CIKs were harvested and co-cultured with DCs sensitized to either AE36 or AE37, in the ratio of 1: 25. The supernatant of the co-culture medium was collected to detect IL-12 and IFN- $\gamma$  with ELISA kits, according to the manufacturer instruction.

## CTL experiment

In the cytotoxicity experiment, CIKs or the CIKs activated by DCs sensitized to AE36 or AE37 were co-cultured with MCF-7 or HeLa cells, and lactate dehydrogenase activity was detected

to evaluate cytotoxicity. MCF-7 or HeLa cells were seeded into a 96-well plate at a density of  $10^4$  cells per well. The effector cells and targeted cells were mixed in different proportions (10:1, 30:1, 50:1) and then co-cultured for 4h at 37°C in 5% CO<sub>2</sub>. The 96-well plate was centrifuged at 1000 g for 5 min. We pipetted 100 µL supernatant from each well to Beckman ready caps to determine the LDH activity of cell release, IL-12 and IFN- $\gamma$  levels. The targeted cells cultured in the medium or Triton X-100 (1% wt/vol) without effector cells were used to detect spontaneous or total LDH release. The results were expressed as specific lysis percentage, calculated as:  $[(LDH_{test} - LDH_{spont}) / (LDH_{total} - LDH_{spont})] \times 100$ . The supernatant of the culture wells with effector and targeted cells in the ratio of 50:1 was taken to detect IL-12 and IFN- $\gamma$  levels with the ELISA kit, according to the manufacturer instructions.

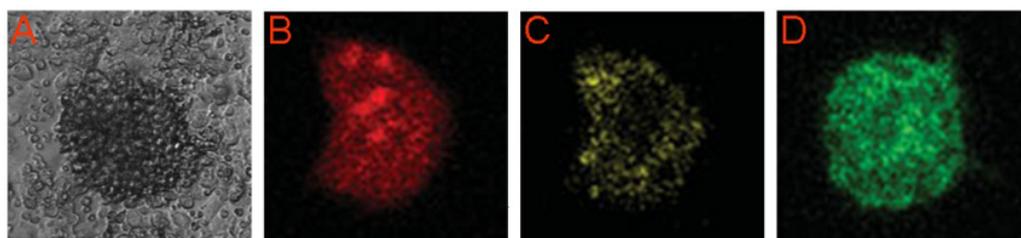
### Statistical analysis

All data were analyzed with the SPSS 11.0 software package (SPSS Inc., Chicago, IL, USA), and expressed as mean  $\pm$  SD; the group difference was compared using the *t*-test.  $P < 0.05$  was considered significant.

## RESULTS

### DC culture and antigen loading

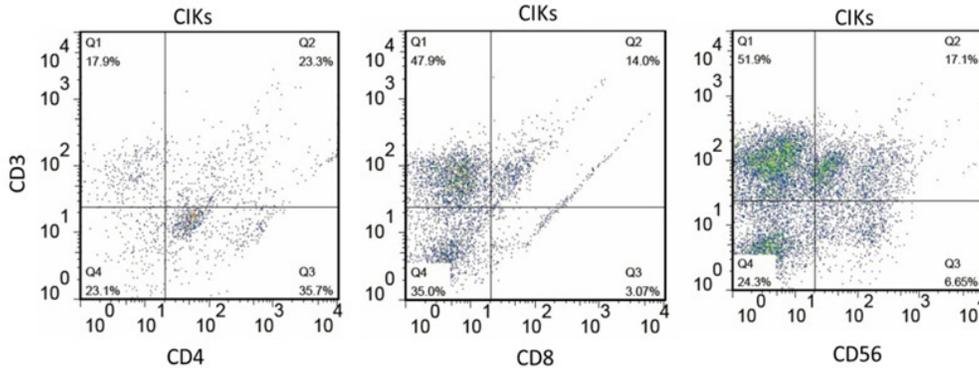
DCs induced by cytokines IL-4 and GM-CSF form typical dendritic cells. FITC-AE37 and Cy5-AE36 were added to the cultured DCs; Cy5-AE36 was added first and after 12 h, FITC-AE37 was added. We observed that under a fluorescence microscope, the red fluorescence decreases and even disappears, while green fluorescence increases and is visible on most of the cell surface (Figure 1). These results demonstrate that AE37 can "hijack" AE36 complexed with MHC, and there is no need for DCs to process the anchored antigen. However, DCs can directly combine with anchored antigen, significantly increasing the antigen loading efficiency of DCs.



**Figure 1.** MHC combination of AE36, AE37, and DCs. **A.** DCs under an ordinary optical microscope. **B.** Combination of Cys-AE36 and DCs. **C. D.** DCs first combined with Cy5-AE36; 12 h later, FITC-AE37 was added. Another 12 h later, DCs were observed under a fluorescence microscope.

### CIK culture and determination of cell surface markers

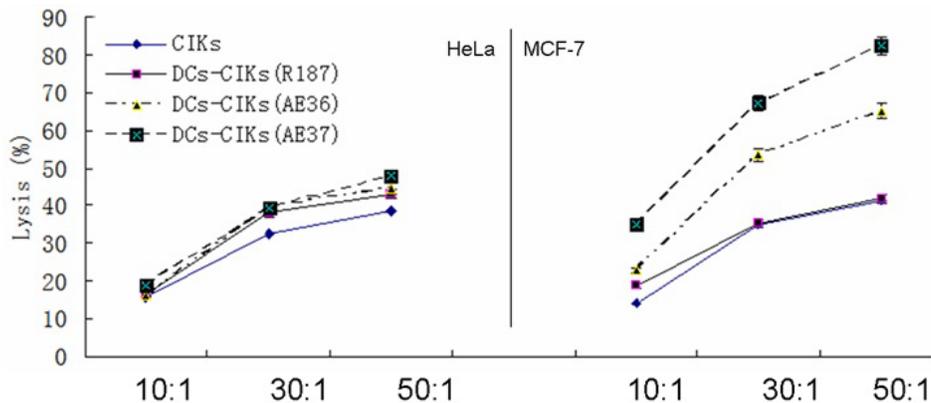
After 14 days of treatment with cytokines (IL-1 $\alpha$ , anti-CD3 antibody, IFN- $\gamma$ , and IL-2), CIKs proliferated greatly. Based on flow cytometry analysis, heterogeneous groups like CD4<sup>+</sup>, CD8<sup>+</sup>, and CD56<sup>+</sup>T were present on CIKs (Figure 2).



**Figure 2.** CIKs analyzed by flow cytometry. CIKs are suppressed cell population, including the CD4+, CD8+, and CD56+ T cells.

### CTL experiments

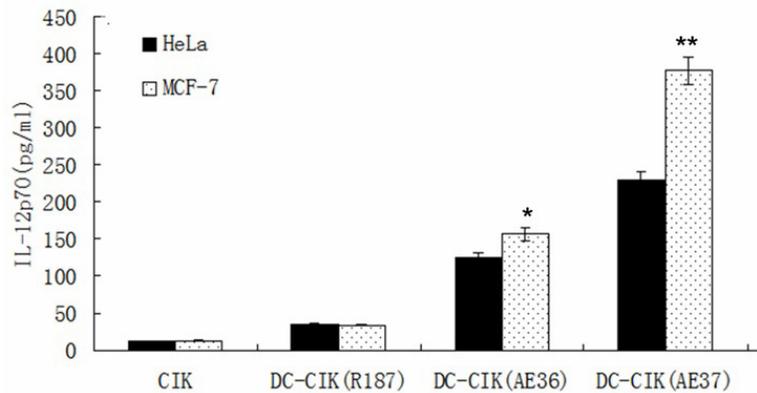
Figure 3 shows the LDH activity of breast cancer or HeLa cells after co-incubation with CIKs, DC-CIK (AE37), DC-CIK (AE36), and DC-CIK (R837). DC-CIK(AE37), DC-CIK (AE36), and DC-CIK (R837) co-cultures have no significant difference in LDH release when present in the ratios of 10:1, 30:1 or 50:1 to HeLa cells, as compared with CIK co-cultures.



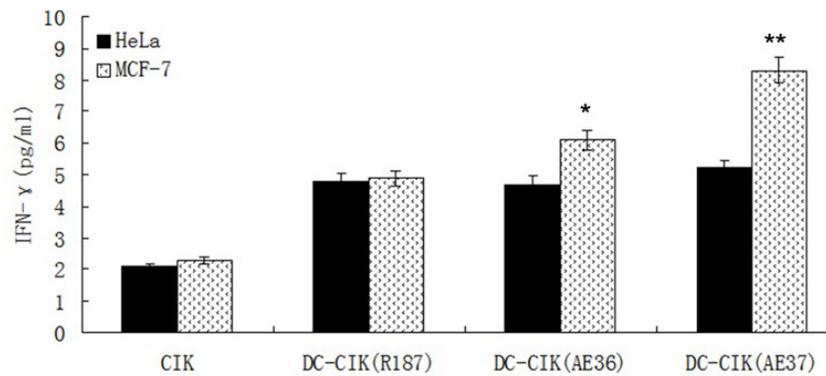
**Figure 3.** CTL experiment with CIK or DC-CIK co-cultured with HeLa or MCF-7 cells.

As the proportion of effector cells to target cells increases, the cytotoxicity of CIKs, DC-CIKs (AE37), DC-CIKs (AE36), and DC-CIKs (R837) also increases. This indicates that the killing of target cells is due to the increase of CIKs and not related to the antigen pulse of DCs. DC-activated CIKs exhibit higher toxicity against HeLa cells compared to pure CIKs. DC-CIKs (AE37) have significantly higher cytotoxicity against MCF-7 than other groups ( $P < 0.01$ ). DC-CIK(AE36) also has higher cytotoxicity than CIKs and DC-CIK (R837), while CIKs and DC-CIK (R837) differ insignificantly in MCF-7 killing activity. These results demonstrate that DC-CIK (AE37) and DC-CIK (AE36) have specific killing activity against MCF-7 cells, which express Her2+. Additionally, we can conclude that the killing effect of DC-CIK (AE37) is greater than that of DC-CIK (AE36), possibly

because AE37 has higher anchoring efficiency than AE36, allowing the sensitized DCs to activate more CD8<sup>+</sup>T cells. We used ELISA kit to detect IL-12 and IFN- $\gamma$  levels in the supernatant of the co-cultures (Figures 4 and 5). The results show that all DC-CIK groups secrete more IL-12 and IFN- $\gamma$  than the CIK group ( $P < 0.01$ ), and DC-CIK (AE36) or DC-CIK (AE37) secrete more IL-12 and IFN- $\gamma$  when killing MCF-7 than HeLa cells ( $P < 0.05$ ). This demonstrates that the interaction of DCs and CIKs promote cells to secrete more IL-12 and IFN- $\gamma$ . In the co-culture of antigen-sensitized DCs and CIKs, more IL-12 and IFN- $\gamma$  were secreted during the process of killing antigen-positive targeted cells; anchored epitope peptide-sensitized DCs also secrete more IL-12 and IFN- $\gamma$  than the CIKs activated by non-anchored epitope peptide when killing epitope peptide-positive MCF-7. A high level of IL-12 and IFN- $\gamma$  promotes anti-tumor activity.



**Figure 4.** IL-12 detected by ELISA in the supernatant of the CIK or DC-CIK and tumor co-cultures. \* $P < 0.05$ ; \*\* $P < 0.01$ .



**Figure 5.** IFN- $\gamma$  detected by ELISA in the supernatant of the CIK or DC-CIK and tumor co-cultures. \* $P < 0.05$ ; \*\* $P < 0.01$ .

## DISCUSSION

Breast cancer prevalence is dramatically increasing in China, mainly due to rapid urbanization and lifestyle changes. Currently, immune therapies for breast cancer are an important part of breast cancer treatment. However, novel and more efficient treatment options need to be

developed. AE37 has been tested in phase I and II clinical trials as a potential therapeutic vaccine against breast and prostate cancers (Holmes et al., 2008; Perez et al., 2010). The research indicates that AE37 is safe and effective and inhibits the growth of Her2+ and ++ breast cancer. However, the studies have not investigated the therapeutic effects of DCs with AE37 directing anchoring culture *in vitro* or the co-culture of sensitized DCs and effector cells on breast cancer.

DCs need to be antigen-sensitized to become DC vaccines or specific activated effector cells (Kalinski, 2009; Palucka and Banchereau, 2013). Ordinarily, for their antigen-sensitization, DCs are required to absorb, process, and present the antigen. As cell culture medium contains other proteins *in vitro*, and DCs are not selective to these proteins, the antigen loading efficiency is affected. This is possibly why DC vaccines or DC-effector cells have been ineffective as therapies. In this study, AE37 was used as the pulse antigen. As AE37 contains the li-Key partial sequence, LRMK, and the sequence interacts with the MHC II molecule, it causes the antigen epitope peptide to bind to the MHC antigen-binding cleft. This pushes out the antigen epitope peptide with low affinity, improving the antigen loading efficiency of DCs. CIKs is a group of heterogeneous cells which stimulate PBMCs *in vitro*, including CD4+, CD8+, CD3+CD56+ NK sample cells. As they can be easily and readily cultured, CIKs are often used in the clinical treatment of tumors. They secrete cytokines IFN- $\gamma$ , IL-12, and perforin, which have anti-tumor activity, and are not MHC-limited. DC-CIK treats tumors better than CIKs, because antigen-sensitized DC-CIKs are specific tumor killing cells. In this study, the CTL experiment showed that AE37-sensitized DC-CIKs have a greater killing effect on MCF-7 cells than AE36-sensitized DC-CIKs ( $P < 0.05$ ), while the cytotoxicity against HeLa cells differs slightly between AE37-sensitized DC-CIKs, AE36-sensitized DC-CIKs, and CIKs ( $P > 0.05$ ). These results indicate that CIKs activated with DCs with higher antigen loading can specifically kill targeted cells. Although CD3+CD56+ NK sample cells are currently considered to play an anti-tumor role in CIKs, other cells like NK cells and CD8+T cells cannot be excluded. In the co-culture system of DC-CIK, antigen-sensitized DCs and CD4+T cells interact to activate CD8+T cells. As these cells co-incubate with tumor cells, they result in stronger anti-tumor activity as compared with CIKs (Gao et al., 2014; Han et al., 2014). The detection of IFN- $\gamma$  and IL-12 levels in the co-cultured supernatant also further demonstrates that CIKs activated by anchored antigen-sensitized DCs secrete more IFN- $\gamma$  and IL-12 than the control groups, promoting anti-tumor activity (Palucka K and Banchereau, 2013; Li et al., 2014).

In summary, as the anchoring antigen of DCs, AE37 can directly combine with MHC. This eliminates the need for DCs to process AE37, increasing the antigen loading efficiency of DCs. This in turn promotes the killing activity of effector cells against tumor cells expressing Her. However, the detailed mechanism needs to be investigated in the future.

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