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Lymphotactin enhances the in-vitro immune efficacy of dendritoma formed by dendritic cells and mouse hepatocellular carcinoma cells*

ZHANG Hao (张 浩)†, JIANG Guo-ping (蒋国平), ZHENG Shu-sen (郑树森),
WU Li-hua (吴丽花), ZHU Feng (朱 峰), YANG Zhen-lin (杨振林)

(*First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou 310003, China*)

†E-mail: haozh@zju.edu.cn

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Abstract: Objective: To investigate the in-vitro antitumor immune responses of dendritoma formed by mouse hepatocellular carcinoma (HCC) cells and lymphotactin (Lptn) gene modified dendritic cells (DCs). Method: DCs prepared from mouse bone marrow were genetically modified by lymphotactin adenovirus, and fused with H22 cells by polyethylene glycol (PEG). RT-PCR and ELISA were employed to identify lymphotactin expression at mRNA and protein level. Cell phenotypes and fusion efficiency was detected by FACS. The stimulatory effect of DC on T cells was detected by mixed lymphocyte reaction. The cytotoxicity activity against H22 cells was assayed by LDH method. Results: Lymphotactin could be efficiently expressed by DCLptn/H22 hybridoma. DCLptn/H22 cells could induce potent T cell proliferation effect and generate strong cytotoxic T lymphocyte (CTL) reaction against allogenic H22 cells. Conclusion: Lymphotactin genetic modification could enhance the in vitro immune activity of the dendritoma.

Key words: Lymphotactin, Dendritic cell, Cell fusion, Hepatocellular carcinoma
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INTRODUCTION

Dendritic cells (DCs) are the most potent antigen-presenting cells (APC) that can elicit primary immune responses. DCs express major histocompatibility (MHC) class I, MHC class II, costimulatory and adhesion molecules that provide secondary signals in activation of naïve T-cell population (Banchereau and Steinman, 1998). The evidence for their ability to act as natural adjuvant in the stimulation of specific anti-tumor cytotoxic T lymphocytes (CTL) and the induction of protective and therapeutic anti-tumor immunity is now overwhelming. Many different protocols have been used to achieve anti-tumor immunity by DCs-based vaccination. In the present study, we constructed lymphotactin (Lptn) gene-modified dendritoma formed by fusion of mouse hepatocellular carcinoma (HCC) cells and Lptn gene modified DCs (DCLptn), and investigated its in-vitro immune effects.

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Animals, recombinant viruses and cell lines

Five- to six-week-old female BALB/C (H-2K^d)

mice obtained from the animal resources center of Shanghai Institute for Biological Sciences, China Academy of Sciences (CAS), were maintained in specific pathogen-free conditions and used at the age of 6–8 weeks. The experiments employing the mice were performed in accordance with institutional guidelines. Recombinant mouse lymphotactin (Lptn) adenovirus (AdLptn) and LacZ adenovirus (AdLacZ) were kindly provided by Dr. Cao Xue-tao. The recombinant adenoviruses were propagated in Human Embryonic Kidney 293 (HEK293) cells provided by Dr. Yang Wei, and purified by cesium chloride (CsCL) density gradient centrifugation. The titers of AdLptn and AdLacZ determined by plaque assay on HEK293 cells were 3.6×10^9 plaque-forming units (PFU)/ml and 4.5×10^9 PFU/ml respectively. H22 cells, which had been established as a BALB/C mouse origin HCC cell line, were purchased from China Center for Type Culture Collection. All of the above cells were cultured in RPMI-1640 (H22 cells) or DMEM (HEK293 cells) medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mmol/L glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

Reagents

Recombinant mouse GM-CSF (granulocyte/macrophage colony-stimulating factor), IL-4 (interleukin-4), TNF-α (tumour necrosis factor-α) and mouse lymphotactin ELISA kit were obtained from R&D company. Fluorescence-conjugated rat monoclonal antibodies against murine (CD3, CD80, CD 86, CD40, CD54, CD11C, DEC205, I-Ad, H-2Kd) were purchased from BD Pharmingen. PKH-26, PKH-2 and 50% PEG (polyethylene glycol, 50% PEG/10% DMSO in PBS) were purchased from Sigma. Lactate dehydrogenase (LDH) cytotoxicity assay kit was purchased from Roche.

Generation of DCs from bone marrow

DCs were prepared according to the method described in Homma *et al*.(2001), with modifications. Briefly, bone marrow cells were obtained from the femora and tibiae of female BALB/C mice. Red blood cells were lysed by treatment with 0.84% ammonium chloride solution. After being washed with PBS, the cells were plated in DMEM plus 10% FCS and 10 ng/ml GM-CSF with conjunction of 10 ng/ml IL-4. On day 3, nonadherent granulocytes, T and B cells were gently removed and fresh media were added. On day 5, loosely adherent proliferating DC aggregates were dislodged and re-plated in fresh media supplemented with 50 ng/ml TNF-α. On day 7 of culture, released, nonadherent mature DCs were harvested.

Gene modification of dendritic cells

DCs were washed twice with PBS and resuspended in serum-free RPMI-1640 with AdLptn at a multiplicity of infection 100 (Cao *et al*., 1998). After 1 h of incubation at 37 °C (with gentle agitation every 20 minutes), the cells were washed with PBS and resuspended in RPMI-1640 (1×10^6 ml^{-1}) supplemented with 10% FCS. Twenty-four hours after gene modification, LacZ gene-modified DCs (DCLacZ) were collected for X-gal staining to evaluate gene transfer efficiency, Lymphotactin gene-modified DCs (DCLptn) were collected for phenotypic analysis, mixed lymphocyte reaction assay, cytotoxicity assay and fusion with H22 cells in-vitro. The culture supernatants from DCs or gene-modified DCs were harvested for chemotaxis assay.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was prepared by the Trizol method (Invitrogene) according to the manufacturer's instructions. cDNA was prepared from 1 μg of RNA using a hexanucleotide random primer and SuperScripte™ Moloney murine leukemia virus reverse transcriptase (Life Technologies) in a total volume of 20 μl. A volume of 2 μl was used for PCR amplification. The upstream primer for mouse Lptn was 5'TGG GGA CTG AAG TCC TAG AAG3', and the downstream primer was 5'TTA CCC AGT CAG GGT TAC TGC TGC TGT G3', with the product size of 300 bp. The upstream primer for mouse beta-actin was 5'TGG AAT CCT GTG GCA TCC ATG AAA C3', the down stream primer was 5'TAA AAG CCA GCT CAG TAA CAG TCC G3',

with expected size of 359 bp. PCR reactions were performed by pre-denaturing at 94 °C for 5 min, then amplification for 25 cycles (94 °C for 1 min, 60 °C for 1 min, 72 °C for 2 min), with a final extension for 10 min at 72 °C. The PCR reaction products were separated by 1.5% agarose containing ethidium bromide.

ELISA for measuring lymphotactin in supernatants

Quantitative determination of lymphotactin protein in the supernatants from gene-modified DCs was performed with a commercial "sandwich" enzyme immunoassay kit according to the manufacture's instructions. Briefly, Costar EIA microplates were coated with 100 μl of 2 μg/ml of rat-anti-mouse lymphotactin as capture antibody, incubated overnight at room temperature, and blocked with 1% bovine serum albumin (BSA) in PBS. Next, 100 μl of serially diluted standards or culture supernatant samples were added in triplicate and incubated for 2 h at room temperature. The plates were washed and then incubated with 100 μl of 400 ng/ml biotinylated goat anti-mouse lymphotactin as detection antibody for 2 h at room temperature. After washing, wells were incubated for 20 min in 100 μl of Streptavidin-horseradish peroxidase (HRP) solution, and developed with substrate solution.

Cell fusion

DCLptn were fused with tumor cells at a 3:1 (DCLptn:tumor) ratio using 50% PEG (Soruri *et al*., 2001). In brief, H22 cells were inactivated by 30 μg/ml mitomycin, then washed and mixed with DCLptn. After centrifugation, 1 ml of PEG was added to the cell pellets for 2 min at 37 °C. After which, an additional 10 ml of warm serum-free medium were added to dilute PEG over the next 3 min with continued stirring. The PEG-treated cells were centrifuged at 400×*g* for 5 min, and resuspended with RPMI-1640 medium supplemented with 20% FCS, 10 ng/ml GM-CSF and 10 ng/ml IL-4, and cultured overnight.

To determine the efficiency of cell fusion, H22 cells were stained with PKH-26 (red fluorescence) and DCs were stained with PKH-2 (green fluorescence); the cells stained with the fluorescence dyes were treated with PEG and cultured overnight as described above. At the second day, the above stained cells were analyzed using a FACScan flow cytometer (Becton Dickinson).

Phenotypic analysis

After washing, DC, DCLptn, and H22 cells were resuspended in PBS containing 1% BSA, and stained with fluorescence-conjugated monoclonal antibody (H-2K^d, I-A^d, CD80, CD86, CD40, CD54) or isotype control antibody (Pharmingen) for 30 min at 4 °C. Stained cells were washed and analyzed using FACS.

In-vitro chemotaxis assay

A 96 well chemotaxis chamber (Neuro Probe) was used for a 2 h chemotaxis assay as previously described (Kurt *et al*., 2001). Spleen cells from naïve BALB/C mice were used as effector cells. The bottom wells of the chamber were loaded with supernants of H22, DC, DCLptn, DCLacZ or RPMI-1640 alone, and the upper wells contained 1×10^5 effector cells. Data were obtained by counting five nonoverlapping high power microscopic fields from each well. Cells were considered to be chemoattracted if the chemotactic index (number of cells migrating in experimental well/number of cells migrating in RPMI-1640 medium only)>2.

T-cell proliferation

Spleen cells from naïve BALB/C mice were passed over nylon wool, assayed purity by FACS (The percentage of CD3$^+$ cells near 90%) and used as responder cells at 1×10^5/well in 96-well U-bottom plates. Syngeneic H22, DCLptn, H22+ DCLptn (H22 cells co-cultured with DCLptn at a ratio of 3:1), DC/H22 (H22 cells fused with DC at a ratio of 3:1) and DCLptn/H22 (H22 cells fused with DCLptn at a ratio of 3:1) cells were inactivated by 30 μg/ml mitomycin for 30 min and added to responder cells in varying cell numbers. Cells were cultured in RPMI-1640 medium containing 10% FCS at 37 °C and 5% CO_2 for 4 days. Control wells

contained either the above stimulating cells alone or T cells alone. At the end of the experiment, 20 μl of the dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were added to each well. After incubation for 3 h at 37 ºC, 100 μl of dimethylsulfoxide (DMSO) were then added to each well and the color intensity was assessed with micro plate reader at 570 nm wavelength. Stimulating index (SI)=(stimulating and responder cells mix−stimulating cells control)/responder cells control.

In-vitro cytotoxicity assay

The functional assay of DCLptn/H22 was determined by the cytotoxicity test; the above groups of inactivated cells were co-cultured with spleen T cells separated from naïve BALB/C mice by the method described above at a 1:10 ratio in the presence of 20 U/ml mouse IL-2 for 7 days. The stimulated T cells were isolated using Ficoll gradient centrifugation to remove dead cell debris and used as effector cells in the LDH cytotoxicity assay. H22 cells were used as target cells. All steps were performed according to the manufacturer's instructions. Briefly, after washing the effector and target cells with assay medium (RPMI1640 with 1% BSA), the effector cells were co-cultured with target cells in a 96-well round bottom plate for 6 h at 37 °C; then the plates were centrifuged and the supernatants were transferred to another flat-bottom ELISA plate. One-hundred μl of LDH detection mixture were added to each well and incubated in the dark for 30 minutes at room temperature. Absorbance was measured by an ELISA reader at 490 nm. The spontaneous release of LDH by target cells or effector cells were assayed by incubation of target cells in the absence of effector cells and vice versa, the maximum release of LDH was determined by incubating the target cells in 1% TritonX-100 in assay medium. The percentage of cell mediated cytotoxicity was determined by the following equation: Cytotoxicity (%)=(effectors and targets mix−effectors control-spontaneous)/(maximum−spontaneous)×100.

Statistical analysis

Data were expressed as means±standard deviation (SD). Experiment results were analyzed by using SPSS 10.0 statistical package. Differences among groups were assessed by the student's *t* test. $P<0.05$ was considered statistically significant.

RESULTS

Lptn expression and function assay in gene modified DCs

Lptn mRNA expression in gene-modified DCs was confirmed by RT-PCR analysis. As shown in Fig.1, DC, H22 did not express any detectable Lptn, whereas it was detected in DCLptn and H22Lptn. The results indicated that Lptn gene could be efficiently transduced by adenovirus vector; and that recombinant adenovirus could infect both DC and H22 cells.

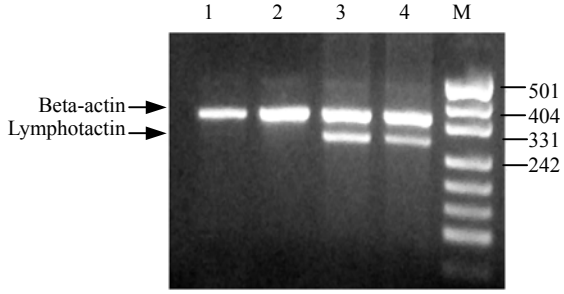

Fig.1 RT-PCR analysis of Lptn mRNA expression in DC, H22, DCLptn, H22Lptn cells (Lane 1−4)

In order to quantitatively determine Lptn protein in supernatants from gene-modified DCs, the culture supernatants were harvested and determined for Lptn production by ELISA. The results showed that about 0.35±0.04 ng/ml Lptn could be detected in the supernatants of DCLptn, while nearly no Lptn could be detected in the supernatants from untransfected DC, DCLacZ and H22 cells.

Consistent with ELISA results, only the supernatants from DCLptn were positive for chemotaxis assay (chemotaxis index=3.2±0.15), but the DC, DCLacZ, H22 groups were negative. The results indicated that the recombinant Lptn secreted from DCLptn showed effective biological activity.

Phenotypic characterization of DCLptn and H22 cells

When being harvested at day 5, the DCs were immature and expressed low levels of I-A^d, CD80, CD86, CD40 and CD54. After being supplied with 50 ng/ml TNF-α and harvested at the 7th day, the DCs showed mature phenotypes, the MHC and costimulatory molecules expression were enhanced. When being modified with AdLacZ and AdLptn, the expression of the above molecules did not change substantially.

Although H22 cells expressed moderate levels of I-A^d, the expression of H-2K^d, CD80, CD86, CD40 and CD54 molecules were almost negative (Fig.2).

Stimulation of T cell proliferation by DCLptn/H22

To determine whether the DCLptn/H22 cells were more effective in stimulating autologous T cells proliferation than DCLptn, H22, DCLptn +H22 or DC/H22 cells. The above groups of cells were co-cultured with autologous T cells. The results showed a higher T-cell stimulation index for DCLptn/H22 (Fig.3). In contrast, although DCLptn +H22, DC/H22, DCLptn cells also stimulated T-cell proliferation to some degree, the stimulation index was much lower than that of DCLptn/H22 (P<0.05). H22 cells had very little stimulatory effect on autologous T cells. These findings demonstrated that DCLptn/H22 was more potent in stimulating autologous T cells.

Enhanced CTL responses induced by DCLptn/H22 dendritoma

To determine the induction of tumor specific CTLs, T cells isolated from naïve BALB/C mice were stimulated by H22, DCLptn, DCLptn+H22, DC/H22 and DCLptn/H22 cells respectively. As shown in Fig.4, T cells incubated with inactivated H22 cells exhibited a low level of target cell lysis. T cells incubated with inactivated DCLptn, DCLptn +H22, DC/H22 cells showed moderate target cell lysis. While T cells stimulated by DCLptn/H22 dendritoma had more potent target cell lysis effect than the above groups (P<0.05).

DISCUSSION

Our objective in this study was to investigate the in vitro immune effects of dendritoma formed by DCLptn and mouse H22 cells. The results presented here clearly demonstrated that DCLptn/H22 cells showed a much greater effect on T cell proliferation and inducing cytotoxic T lymphocytes compared with DCLptn, DCLptn+H22 or DC/H22 cells.

A major reason for the relative ineffectiveness of vaccination in controlling human tumor growth

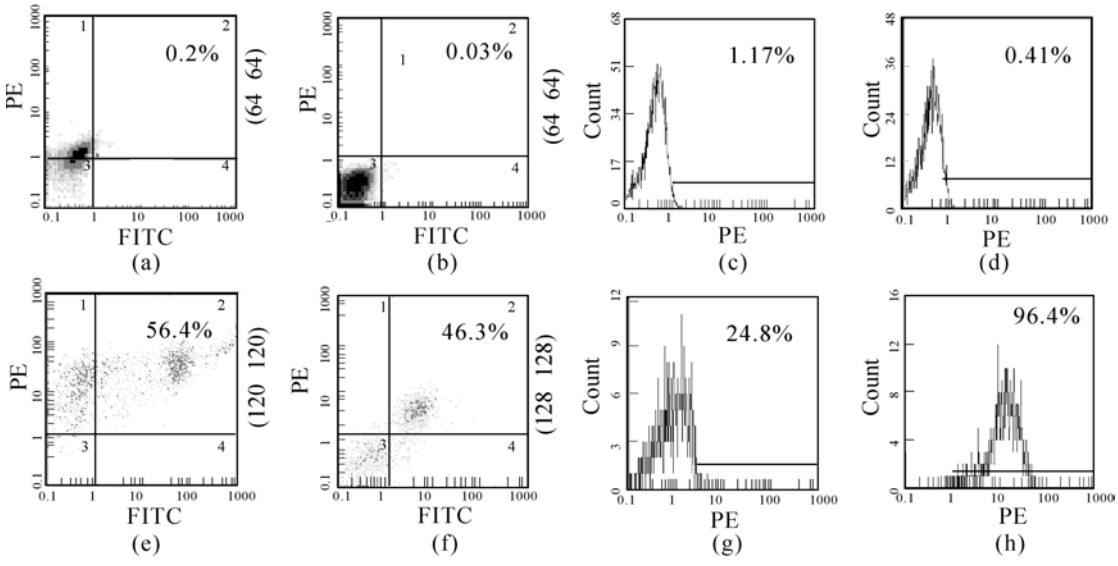

Fig.2 FACS analysis of the phenotypes of H22 and DCLptn cells. (a) H22 H_2-K^d/I-A^d; (b) H22 CD80/86; (c) H22 CD40; (d) H22 CD54; (e) DCLptn H_2-K^d/I-A^d; (f) DCLptn CD80/86; (g) DCLptn CD40; (h) DCLptn CD54

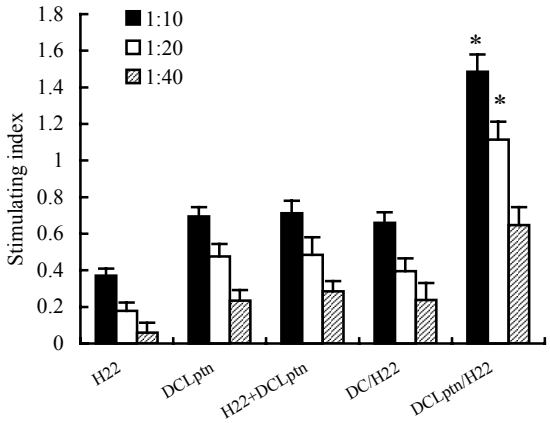

Fig.3 Stimulation of autologous T cells by DCLptn/ H22 cells. The stimulation index is expressed as the mean±SD of 3 experiments, each performed in triplicate. *P<0.05 vs H22, DCLptn, DC/H22 and DCLptn+H22 groups

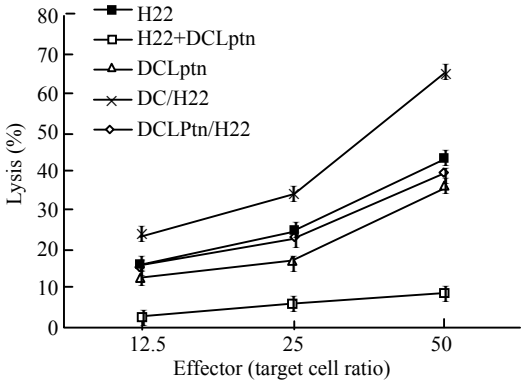

Fig.4 Stimulation of antitumor CTLs by DCLptn/H22 cells. The specific lysis is expressed as the mean±*SD* of 3 experiments each performed in triplicate. *P<0.05 vs H22, DCLptn, DC/H22 and DCLptn+H22 groups

lies in the many mechanisms devised by neoplastic cells to avoid recognition and destruction by the immune system. These mechanisms include changes in the phenotype or gene expression of tumor cells, such as down-regulation of the expression of MHC and costimulatory molecules, the release of immune suppressive factors (e.g. Vascular Endothelial Growth Factor, IL-10, Transforming Growth Factor-β) that can impair the activity of DCs (Moingeon, 2001). So it is the key in tumor immunotherapy to augment the antigen-presenting function of DCs and to improve the microenvironment of antigen presenting.

Our data showed that although H22 cells expressed moderate levels of MHC I, the expression level of CD80, CD86, CD40 and CD54 were very low as in human HCC, so they could not stimulate the proliferation of T cells efficiently. These indicated that low expression of stimulatory molecules could be a main mechanism of immune escape of HCC. In contrast to H22 cells, mature DCs expressed high levels of MHC and costimulatory molecules. But upon maturation, they lost endocytic/phagocytic receptors and had a lower capacity for uptaking of exogenous antigens. So DCs or DCs modified by chemokines or cytokines as stimulator had limited effect in stimulating T-cell proliferation and inducing CTL.

Recent studies demonstrated that fusion of DCs with tumor cells was a novel and promising method in delivering tumor antigens into DCs (Gong *et al*., 2002). The hybrid cells retain important characteristics of APC and tumor cells. These include the endogenous expression of multiple tumor antigens and their presentation in context with high levels of MHC I, MHC II, and costimulatory molecules of the DC partner. Secondly, the fusion of tumor cells with DCs expressing the entire repertoire of tumor antigens (Ags) should allow immunization of the host with multiple tumor Ags without knowing the identity of the tumor Ags (Wang *et al*., 1998). However, despite the crucial immunological importance of dendritoma, it still remains a challenge. In the present study, our data showed that although DC/H22 cells had stronger immune responses than H22 cells in stimulating T-cell proliferation and inducing CTL, the effects in DC/H22, DCLptn and DCLptn+H22 groups were similar. So several parameters must optimized in order to maximize dendritoma immune efficacy in the immunotherapy of cancers.

Chemokines are a superfamily consisting of 8 to 11 KD proteins subdivided into four families (C, CC, CXC, and CX_3C) according to the position and separation of their first two amino-terminal cysteine (c) residues of a four-cysteine motif. Chemokines play an important role in the recruitment and activation of specific subsets of leukocytes. The migration of DC to the sites of inflam-

mation where they capture the antigens and subsequently migrate to the local lymph nodes is regulated by the expression of different chemokines and chemokine receptors. Lymphotactin is a C chemokine produced mainly by T and natural killer (NK) cells, not by DCs and H22 cells. It has also been characterized as a T and NK cell chemoattractant both in vitro and in vivo (Emtage *et al*., 2002; Huang *et al*., 2002; Kurt *et al*., 2001). Lptn gene modification of DC can improve its preferential chemotaxis on T and NK cells and consequently optimize the microenvironment of Ag presentation and favor DC Ag presentation to T cells. So in order to improve the efficacy of dendritoma, we constructed Lptn gene modified dendritoma and investigated its immune effects.

It is now well known that $CD4^+$ and $CD8^+$ T cells have an important role in immune responses. After fusion of dendritic cells with tumor cells, the dendritoma mainly display the antigens through the MHC class I pathway, which stimulates $CD8^+$ T cells. Moreover, they can also present tumor antigens by MHC class II pathway and thereby stimulate $CD4^+$ T helper cells. $mXCR_1$ (mouse X chemokine receptor 1), which is specific receptor of mouse lymphotactin, expressed mainly in $CD8^+$ T cells and NK cells (Yoshida *et al*., 1999). So after Lptn gene modification, the newly activated T cells could acquire more tumor antigens from DCLptn/H22 and showed stronger cytotoxicity to target cells. In addition, the NK cell interactions with DCs also could influence the immunological outcome. After Lptn gene modification, the Lptn/H22 dendritoma could enhance its interaction with NK cells and thus results in substantial increase in both NK cell cytolytic activity and IFN-γ production.

Taken together, our study demonstrated that DCLptn/H22 cells could induce potent T-cell proliferation and strong CTL reaction against allogenic H22 cells. Immunization engineered fusion hybrid vaccines may be an attractive strategy for cancer immunotherapy.

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