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The influence of therapeutic vaccine candidate against HBeAg pEGFP-N1-C (472–507)-ecdCD40L on dendritic cells

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Summary. – Hepatitis B virus (HBV) infection is a major public health problem and immune tolerance is responsible for persistent HBV infection. HBV therapeutic vaccines targeting HBV e antigen (HBeAg) may have an excellent effect in overcoming HBV immune tolerance. Thus, there is urgency for designing therapeutic vaccine candidates that target HBeAg. In this research, we fused the C (472–507) gene sequence of HBV with the extracellular domain of human CD40 ligand sequence and ligated this fused sequence into the pEGFP-N1 vector to construct the recombinant plasmid pEGFP-N1-C (472–507)-ecdCD40L. Then, the dendritic cells (DCs) generated from human peripheral blood were transfected with this recombinant plasmid. After this, the phenotype and function of DCs were assessed. Compared with the three control groups of pEGFP-N1-C (472–507), pEGFP-N1 and phosphate buffered saline (PBS), we found that DCs transfected with the recombinant plasmid pEGFP-N1-C (472–507)-ecdCD40L enhanced the expression of costimulatory molecules (CD80, CD86 and HLA-DR) and secretion of cytokine IL-12p70. Furthermore, the capacity of inducing the proliferation of allogeneic lymphocytes was also improved. Our study validated that transfecting DCs with recombinant plasmid pEGFP-N1-C (472–507)-ecdCD40L could activate DCs and enhance their functions. Therefore, C (472–507)-ecdCD40L fusion sequence may be a promising vaccine candidate for chronic hepatitis B therapythat targets HBeAg.

Keywords: CD40 ligand; dendritic cells; hepatitis B virus e antigens; therapeutic vaccines

Introduction

Hepatitis B is a worldwide disease with 240 million chronically-infected individuals. It results in more than 1 million deaths per year due to complications of chronic hepatitis B (CHB), which include cirrhosis and hepatocellular carcinoma (HCC)(Revill *et al*., 2016). Although a prophylactic vaccine is available, no cure exists for chronically-infected individuals. Currently, the available treatment methods for CHB include pegylated interferon (PEG-IFN) alpha and nucleos(t)ide analogues (NAs). These treatments only achieve viral suppression and lifelong therapy is necessary in the majority of infected persons. However, the side effects of PEG-IFN and drug resistance of NAs limit their clinical usage (Kang *et al*., 2015; Trépo *et al*., 2014). There are several antiviral strategies that have been explored in recent years that include directly-acting antivirals (DAAs) and host-targeting agents (HTAs). The DAAs include HBV new polymerase inhibitors, entry inhibitors, agents targeting covalently-closed circular DNA (cccDNA), agents targeting HBV capsids, inhibition of

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Abbreviations: HBV = hepatitis B virus; CHB = chronic hepatitis B; PEG-IFN = pegylated interferon; NAs = nucleos(t)ide analogues; DCs = dendritic cells; HBeAg = hepatitis B virus E antigen; ecd-CD40L = exocellular domain of human CD40 ligand; HCC = hepatocellular carcinoma; TLR = toll-like receptor

HBV gene expression and cyclophilin inhibitors. The HTAs include innate immune ligands, check-point inhibitors, cellular inhibitor of apoptosis protein inhibitors and therapeutic vaccines (Lin and Kao, 2016; Petersen *et al.*, 2016). However, these strategies are under investigations and not available for clinical usage. Therefore, we need to urgently search for a novel method to eliminate the virus or achieve persistent suppression of the virus.

CHB is characterized by an impaired HBV-specific immune response, especially the exhaustion of virus-specific T-cells (Maini and Schurich, 2010). The current immunomodulatory therapy of CHB aims to overcome immune tolerance and multiple studies have made progress in this direction. One study proved that DCs stimulated by HBV subviral particles *in vitro* and used to immunize mice can induce HBV-specific immune responses (Farag *et al.*, 2012). Another study immunized HBV transgenic mice with toll-like receptor (TLR) 7/8 agonists conjugated with HBV-Ag and reversed their immunotolerant state (Wang *et al.*, 2014). Some studies have attempted to overcome immune tolerance by optimizing vaccine formulation (Backes *et al.*, 2016). However, these strategies were effective in only a fraction of studies and the most effective therapy should consider many strategies simultaneously. Therefore, it is necessary to identify novel strategies to overcome immune tolerance.

The relationship between the HBeAg and persistent HBV infection has been illustrated by multiple studies. One study indicated that HBeAg could suppress specific cellular immunity to clear the virus by reducing IFN-γ produced by T-lymphocytes, increasing Th2-type cytokine secretion, upregulating B7-H1 and downregulating TLR2 on monocytes (Han *et al.*, 2013). Another study illuminated that HBeAg was able to upregulate TLR3, TLR4 and PD-1 expression, while decreasing IFN-γ production by lymphocytes. The proliferation of lymphocytes also could be inhibited by HBeAg (Chen *et al.*, 2017). Our previous studies also found that HBeAg has a negative effect on the maturation of DCs, which might be responsible for a persistent infection of HBV (Lan *et al.*, 2016a; Lan *et al.*, 2016b). Our other study indicated that the high-dose HBeAg could induce DCs to differentiate into regulatory DCs in mice (Wu *et al.*, 2016). Therefore, overcoming the HBV immune tolerance through eliminating HBeAg is a feasible strategy. The humoral immunity against HBeAg, which had emerged as HBeAg/HBeAb seroconversion, could be induced by NAs or PEG-IFN (Kao, 2014). However, the key of HBV immune tolerance is the exhaustion of specific T-cells (Maini and Schurich, 2010) and a method aiming to induce cellular immunity targeting HBeAg has not been reported. Thus, the strategy aiming to induce cellular immunity against HBeAg could be a promising therapeutic method.

HBV therapeutic vaccination is a promising new strategy for inducing cellular and humoral immunity, which has made great progress in recent years (Michel *et al.*, 2011). The peptide of HBeAg and HBcAg (PPAYRPPNAPIL) is immunogenic Th-cell recognition site and is non-tolerogenic (Milich *et al.*, 1989; Chen *et al.*, 2005). Furthermore, this peptide is encoded by the C gene of HBV, the sequence of corresponding gene region in C (472–507) is 'CCTCCAGCT TATAGACCACCAAATGCCCCTATCTTA'. Thus, we chose the C (472–507) sequence to construct the candidate for the therapeutic vaccine. A previous study has proven that fusing the vaccine sequence with the extracellular domain of human CD40 ligand (ecdCD40L) gene could promote the activation of DCs and enhance their function (Wu *et al.*, 2011). Therefore, with the purpose of investigating the ability of this Th-cell recognition site to activate DCs, we fused the C (472–507) sequence with CD40L gene and ligated this fused sequence into the pEGFP-N1 vector to construct recombinant plasmid pEGFP-N1-C (472–507)-ecdCD40L. Following this, the DCs generated from human peripheral blood were transfected with this recombinant plasmid. Then, the phenotype and function of DCs were detected. Through this study, we examined the influence of C (472–507)-ecdCD40L sequence on DCs, which is essential for designing HBV therapeutic vaccine targeting HBeAg.

Materials and Methods

Construction of recombinant plasmid. We used three PCR steps to fuse the C (472–507) sequence and ecdCD40L gene. The first PCR step aimed to amplify the C (472–507) sequence 'CCTCCAGCT TATAGACCACCAAATGCCCCTATCTTA' and ecdCD40L gene. Briefly, the C (472–507) sequence was cloned by PCR. Two primers specific for the C (472–507) sequence were used, which were namely the primer A (5'-GCTAGCGATGCCTCCCG-3') and primer B (5'-TTCTATGAAGGCCGCC-3'). The cDNA fragment coding for the full open reading frame of human ecdCD40L genewas cloned by reverse transcription polymerase chain reaction (RT-PCR). Two primers specific for ecdCD40L gene were used, which were namely primer C (5'-CGGCGGCGGCCTTCATAGA-3') and primer D (5'-GGATC CCGGAGTTTGAGTAAGC-3'). The second PCR step aimed to fuse the C (472–507) sequence and ecdCD40L gene. The PCR products of two sequences were retrieved and added to the same system for PCR, with no primer used. The third PCR step aimed to amplify the fusion sequence C (472–507)-ecdCD40L, the products of the second PCR step were used as templates and amplification was performed using primers A and D. The PCR procedure was as follows: 94°C for 5 min, 94°C for 30 s, 56.4°C for 30 s, 72°C for 50 s and 72°C for 7 min after 30 cycles. The products of third PCR step were ligated into the pEGFP-N1 vector by T4 DNA ligase (TaKaRa) to construct the recombinant plasmid pEGFP-N1-C (472–507)-ecdCD40L. The recombinant plasmids were then confirmed by restriction endonuclease digestion (Fermentas) using restriction enzymes *Hind*III/*Bam*HI.

Generation of dendritic cells. Human peripheral blood was donated by healthy adults. All donors gave written informed

consent prior to inclusion. Furthermore, all of the protocols were in compliance with institutional guidelines and approved by the ethical guidelines of the declaration of Helsinki as reflected in prior approval by the institution's human research committee. The peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation. Subsequently, PBMCs were centrifuged on a Percoll gradient, which consisted of three layers. The light density fraction containing monocytes was seeded in 12 well-culture plates at a density of 1×10^6 cells/ml. After 3 h of incubation at 37°C, non-adherent cells were removed and adherent cells were only from the population of desired DCs, which were cultured in the complete culture medium [RPMI-1640 as well as 15% fetal calf serum (FCS), GM-CSF (100 ng/ml) and IL-4 (50 ng/ml)] for 5 days.

Transfection. After 5 days in the culture, DCs were transfected with the recombinant plasmid pEGFP-N1-C (472-507)-ecdCD40L (group A) using Lipofectamine™ 2000 reagent (Invitrogen), following the manufacturer's guidelines. pEGFP-N1-C (472–507) (group B), pEGFP-N1 (group C) and PBS (group D) were used as controls. After 48 h, the efficiency of transfection was examined using the fluorescence microscope.

Flow cytometry. To quantify the expression of surface molecules on DCs, DCs were collected from 12 trans-well plates 48 h after transfection, before being washed with PBS. A total of 1×10^5 DCs were stained for 30 min on ice with PE- or FITC-conjugated monoclonal antibodies (mAb) to CD80, CD86 and HLA-DR (eBioscience). Isotype-matched antibodies served as FITC- or PE-conjugated controls (eBioscience). The cells were then washed twice with PBS and analyzed by flow cytometry (Becton Dickinson FACS-calibur).

Measurement of IL-12 levels. The supernatant of DCs was collected 48 h after transfection with pEGFP-N1-C (472–507)-ecdCD40L, pEGFP-N1-C (472–507), pEGFP-N1 and PBS. The concentration of IL-12p70 was detected by ELISA using corresponding ELISA kits (R&D Systems, Minneapolis, MN).

Allogeneic mixed lymphocyte reactions (MLRs). To assess the ability of C (472–507)-ecdCD40L-modified DCs to induce T-cell proliferation, T-cells were collected from human peripheral blood mononuclear cells and used as effector cells. Graded doses of DCs were co-cultured with constant numbers (1×10^5) of allogeneic T-cells with different stimulator/responder (DC/T-cell) ratios (1:5, 1:10, 1:20) for 96 h in 96-well plates in a total volume of 200 µl. The T-cell proliferation was expressed as stimulation index (SI) value and measured using the CCK-8 cell proliferation assay kit (Beyotime Institute of Biotechnology, Haimen, China) in accordance with the manufacturer's instruction.

Statistical analysis. All data were reported as means ± standard deviations (S.D.). Statistical analysis was performed by one-way ANOVA followed by post-hoc tests (using LSD-t or Dunnett's T3) for multi-group comparisons with the SPSS15.0 program. All *P*-values <0.05 were considered significant.

Results

Construction of recombinant plasmids

As shown in Fig. 1a, the PCR products had the expected molecular weight (C (472–507)-ecdCD40L fusion sequence:

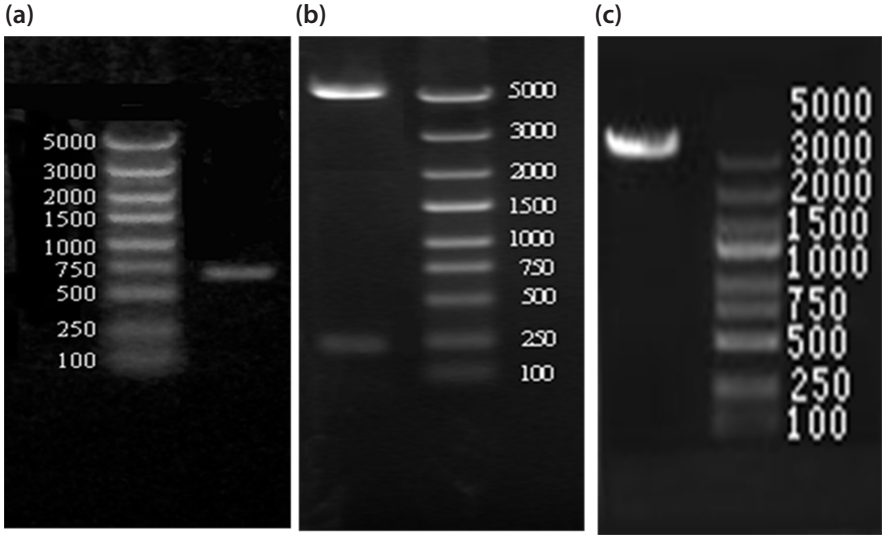

Fig. 1

The DNA electrophoresis of fragments

(a) C (472–507)-ecdCD40L fusion sequence: 714 bp; **(b)** Combinant plasmid pEGFP-N1-C (472–507)-ecdCD40L was digested by HindIII/BamHI, the fragments were approximate 241 bp and 5131 bp; **(c)** Combinant plasmid pEGFP-N1-C (472–507) was digested by *Eco*R I, the fragment was approximately 4742 bp. The experiment was performed independently three times.

Table 1. The expression of co-stimulation molecules of dendritic cells

Group	CD86 (%)	CD80 (%)	HLA-DR (%)
A	98.08±1.42[a]	88.62±3.51[a]	81.16±5.23[a]
B	93.30±2.60	76.57±7.03	77.43±2.60
C	90.35±2.16	74.12±5.64	70.75±2.71
D	91.59±1.92	66.98±6.61	71.50±5.07

[a]Group A compared with other three groups, P <0.05. Group A: DCs transfected with pEGFP-N1-C (472–507)-ecdCD40L; group B: DCs transfected with pEGFP-N1-C (472–507), group C: DCs transfected with pEGFP-N1; group D: DCs transfected with PBS.

Table 2. Spontaneous IL-12p70 level in pure DCs population (pg/ml)

	A	B	C	D
IL-12p70 (pg/ml)	170.50±18.88[a]	122.50±20.04	109.50±17.14	106.00±19.53

[a]Group A compared with other three groups, P <0.05. Group A: DCs transfected with pEGFP-N1-C (472–507)-ecdCD40L; group B: DCs transfected with pEGFP-N1-C (472–507), group C: DCs transfected with pEGFP-N1; group D: DCs transfected with PBS.

714 bp). The C (472–507)-ecdCD40L fusion sequence was cloned into the vector pEGFP-N1 to construct the recombinant plasmid pEGFP-N1-C (472–507)-ecdCD40L. After this, it was characterized by digestion with restriction enzymes *Hind*III/*Bam*HI. The fragments digested from the recombinant plasmids by *Hind*III/*Bam*H I were approximately 241 bp and 5131 bp, as expected (Fig. 1b). Meanwhile, the C (472–507) sequence was cloned into the vector pEGFP-N1 to construct the recombinant plasmid pEGFP-N1-C (472–507), before being characterized by digestion with restriction enzyme *Eco*RI. The size was approximately 4742 bp, as expected (Fig. 1c).

Morphological characteristic

The progenitor cells of DCs were incubated in a complete medium containing GM-CSF and IL-4 at 37°C in 5% CO_2 atmosphere. Initial DCs were circular and adherent cells. Furthermore, proliferation and increase in size of DCs could be observed from the third day. Gradually, the DCs displayed the typical morphology of many dendrites under phase-contrast microscopy (data not shown).

Cell surface phenotype

To examine the phenotypic changes induced within DCs after being transfected with pEGFP-N1-C (472–507)-ecdCD40L, four groups of transfected DCs were subjected to phenotypic analysis by flow cytometry. Our data showed that compared with the three control groups, DCs transfected with pEGFP-N1-C (472–507)-ecdCD40L upregulated expression of immunologically important cell surface molecules (CD80, CD86 and HLA-DR), indicating a significant difference (Table 1). The experiment was independently performed three times.

IL-12p70 production by DCs

IL-12 is a cytokine produced by DCs and is able to induce T-cell proliferation. Therefore, levels of IL-12 can be used as an important index to evaluate the functions of DCs. Compared with the three control groups, DCs transfected with pEGFP-N1-C (472–507)-ecdCD40L upregulated cytokine IL-12p70 production, indicating a significant difference (Table 2). The experiment was independently performed three times.

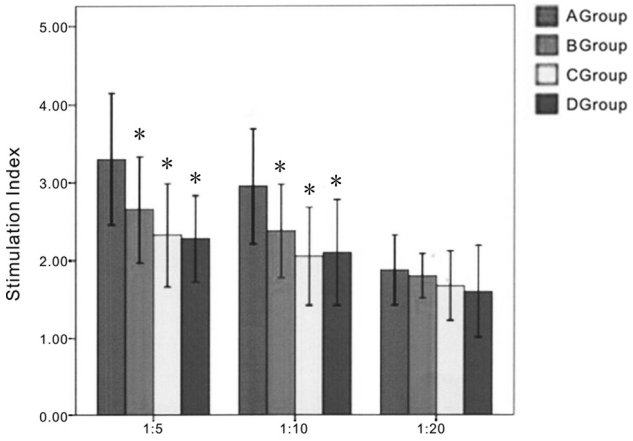

Fig. 2

The capacity of dendritic cells to stimulate lymphocyte proliferation
DCs function was analyzed in anallogeneic mixed lymphocyte reactionby incubating DCstransfected with the recombinant plasmid or PBS with allogenic T lymphocytes at the indicated ratios. Group A: pEGFP-N1-C (472–507)-ecdCD40L; Group B: pEGFP-N1-C (472–507); Group C: pEGFP-N1; Group D: PBS. The experiment was performed independently three times.

Allogeneic T-cell proliferation

Allogeneic mixed lymphocyte reactions demonstrated that DCs transfected with pEGFP-N1-C (472–507)-ecd-CD40L were more potent stimulators of allogeneic T-cells compared to the controls. Results showed that when the DC/T-cell ratio was 1:5 or 1:10, DCs transfected with pEGFP-N1-C (472–507)-ecdCD40L induced the highest proliferative response compared with the other groups, indicating a significant difference ($P < 0.05$). When DC/T-cell ratio was 1:20, there were no significant differences between the four groups in stimulating allogeneic lymphocyte proliferation ($P > 0.05$) (Fig. 2).

Discussion

We have constructed pEGFP-N1-C (472–507)-ecdCD40L eukaryotic expression vector and examined the alteration of DCs transfected with this recombinant plasmid. Compared with the controls, we found that DCs transfected with pEGFP-N1-C (472–507)-ecdCD40L could upregulate the expression of costimulatory molecules (CD80, CD86 and HLA-DR) and production of proinflammatory cytokines (IL-12p70). Furthermore, the capacity to induce allogeneic lymphocytes proliferation was enhanced. It also supported the concept that genetic modification of DCs with a recombinant C (472–507)-ecdCD40L might be a useful strategy for activating DCs and enhancing their functions, which is essential when designing a HBV therapeutic vaccine targeting HBeAg.

The HBV therapeutic vaccine has undergone considerable development in recent years. A HBV therapeutic vaccine based on autophagosomes could suppress HBV replication and break HBV tolerance, and finally clear the HBV-infected hepatocytes in mouse models (Xue *et al.*, 2014). A study constructed a DNA vaccine, which had the ability to induce robust T-cell and humoral responses in both mice and human patients (Yoon *et al.*, 2015). Another HBV polytope DNA vaccine constructed of surface antigen epitopes was found to have the ability to inhibit HBV DNA replication and downregulate surface antigens in HBV transgenic mouse models (Li *et al.*, 2005). A research proved that the vaccine Nasvac formulated of surface and core antigens was capable of stimulating both B- and T-cells *in vitro* (Lobaina *et al.*, 2015). As for the vaccine adjuvant, a study demonstrated that GM-CSF could be used as an immune adjuvant for both preventative and therapeutic purposes (Qing *et al.*, 2010). However, there is no available therapeutic vaccine against HBV in clinical medicine and the effect of HBV therapeutic vaccines should be further determined. In chimpanzees chronically infected by HBV models, DNA vaccines encoding HBsAg+PreS2+HBcAg and IL-12 failed to control HBV viremia (Shata *et al.*, 2006). A clinical study demonstrated that the HBV plasmid DNA (pSG2.HBs) vaccine had no effect on controlling the HBV infection despite being well-tolerated (Cavenaugh *et al.*, 2011).

To sum up, numerous HBV therapeutic vaccines have been reported, but none of them had been as successful as initially anticipated for CHB. In addition, none of them decreased the HBeAg level in order to overcome immune tolerance. The impairment of the immune responses, especially the exhaustion of T-cells generated during persistent HBV infection, results in them not responding appropriately to the therapeutic vaccination, and is probably responsible for the poor responses after therapeutic vaccines (Michel *et al.*, 2011). The HBeAg has a negative effect on immune system and reduction of the HBeAg level is a feasible approach to improve the response after therapeutic vaccination. Therefore, a therapeutic vaccine targeting the HBeAg may be a promising strategy to overcome the immune tolerance in CHB.

CD40 is a member of the tumor necrosis factor receptor superfamily and the CD40-CD40L interaction plays an important role in the maturation and function of DCs (Ma and Clark, 2009), which provides us a feasible way to design vaccines targeting DCs. A recent study reported that activating CD40-CD40L pathway could induce rapid accumulation of vaccine-induced T-cell responses in Mycobacterium tuberculosis infections (Griffiths *et al.*, 2016). Another study demonstrated that transferring CD40L gene could induce tumor suppression by activation of DCs (Serba *et al.*, 2008). Previous study had proved that the HBV S-ecdCD40L fusion gene could promote the activation and enhance the function of DCs (Wu *et al.*, 2011). Therefore, in this study, we constructed recombinant plasmid pEGFP-N1-C (472–507)-ecdCD40L and tested its function in activating DCs. The strategies to improve the effectiveness of vaccination by targeting DCs have been utilized in recent years (Kastenmuller *et al.*, 2014). Many vaccines against infectious diseases were often associated with an adjuvant to activate DCs, which can significantly enhance the effectiveness of vaccinations (Palucka *et al.*, 2010).

The maturation of DCs is characterized by high expression of CD80, CD86 and HLA-DR. Furthermore, the production of IL-12 by DCs is critical for the maturation of T helper 1 (Th1) cells and the development of cell-mediated immunity. A report demonstrated that the function of DCs depends on the expression of costimulatory molecules, antigens and secretion of IL-12 (Kaka *et al.*, 2008). In addition, IL-12 reduces the probability of cancer development in CHB, which is a common cause of death in CHB. A study indicated that microRNA-21 could inhibit expression of IL-12, thereby increasing the proliferation of HCC (Yin *et al.*, 2016). Another study demonstrated that genetic variants in IL-12 were associated with an increased risk of HBV-related HCC (Tan

et al., 2016). Yet another study suggested that administration of IL-12 directly into tumors might represent a strategy for treatment of CHB and associated HCC (Rodriguez-Madoz *et al.*, 2009). Therefore, in this study, we examined IL-12 p70 secretion levels of DCs by ELISA 48 h after transfection with our recombinant plasmids and detected the expression of CD80, CD86, HLA-DR on DCs by flow cytometry. The results showed that compared with controls, DCs transfected with pEGFP-N1-C (472–507)-ecdCD40L upregulated expression of co-stimulatory molecules (CD80, CD86 and HLA-DR) and production of cytokine IL-12p70. The results suggested that pEGFP-N1-C (472–507)-ecdCD40L transfection could promote the maturation and function of DCs.

The ability of DCs to stimulate T-cells is also an effective indicator of the immunogenicity of vaccines. In our study, DCs and lymphocytes were co-cultured 48 h after transfection, with results showing that when DC/T was 1:5 or 1:10, DCs transfected with pEGFP-N1-C (472–507)-ecdCD40L had significantly increased T-cell stimulatory activity ($P < 0.05$) compared with controls. The result suggested that the C (472–507)-ecdCD40L fusion sequence could activate DCs and induce a stronger specific immune response. However, when DC/T was 1:20, there was no significant difference between the four groups, which may be due to the amount of DCs not being enough to promote T-cell proliferation. In this study, we constructed the pEGFP-N1-C (472–507)-ecdCD40L eukaryotic expression vector and examined the alteration of DCs transfected with recombinant plasmid pEGFP-N1-C (472–507)-ecdCD40L. This is necessary in designing a HBV therapeutic vaccine targeting HBeAg and reducing the probability of cancer development in CHB. However, the effectiveness of this vaccine candidate in suppressing viral load should be further determined *in vivo*. In forthcoming research, we will inject the HBV transgenic mice with this vaccine candidate and detect the viral level in mice. Eventually, we hope to prove that this vaccine candidate indeed is capable of suppressing or eliminating HBV.

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