Adjuvant Effects of Dual Co-stimulatory Molecules on Cellular Responses to HIV Multiple-epitope DNA Vaccination

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Abstract Designing adjuvants that can induce strong cytotoxic T cell responses is largely required for preparing DNA vaccines. In this study we explored dual costimulatory molecules 4-1BBL and OX40L as adjuvants to improve the efficiency of the HIV multiple-epitope DNA vaccine. When explored in the human dendritic cell-T cell based co-culture system, dual costimulatory molecules significantly enhanced the anti-HIV T cell response of the HIV multiple-epitope DNA vaccine, as detected by intracellular cytokine staining to HIV antigens, cytokines accumulation in the cultures, and antigen-specific cytotoxic T lymphocyte responses. These results suggest that dual costimulatory molecules 4-1BBL and OX40L can effectively increase the potential of the HIV multiple-epitope antigen DNA vaccine and may provide an exciting approach for HIV therapy.

Keywords HIV DNA vaccine; Co-stimulatory molecules; 4-1BBL; OX40L; Dendritic cells
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1 Introduction

Development of an effective vaccine against HIV is urgently required nowadays. DNA-based vaccines, which can induce cell mediated immunity against infected cells, therefore, may be a promising therapeutic strategy for defensing HIV infection. However, DNA vaccines commonly induce weak immune responses in humans[1], a new adjuvant method has been proposed to increase efficacy of DNA-induced immune response.

OX40(CD134) is a 50-kDa transmembrane protein of TNFR superfamily and exerts a role in either enhancing or bypassing CD4 T cell help in eliciting potent antiviral CTL responses. OX40L, the ligand of OX40, is expressed on mature-activated APCs such as dendritic cells(DCs) and macrophages[2]. OX40/OX40L signaling has been illustrated to enhance the capacity of CD4 T cells in proliferating, and producing IL-2, to inhibit the apoptosis of CD4 T cells *via* increased Bcl-2/Bcl-x expression, and to reverse T cell anergy. 4-1BB(CD137) is another important costimulatory molecule and functions in activated T cells, NK cells, and monocytes[3]. Its ligand, 4-1BBL, is expressed on the activated APCs such as DCs, macrophages, and B cells[4]. The effects of 4-1BB/4-1BBL signaling has been shown to play a central role in human antiviral CD8 T cell responses of the primary immune response, resulting in enhanced expansion and differentiation of mature effector cells with high levels of perforin, granzyme A, and cytolytic activity[5]. 4-1BB is also important to regulate the secondary immune response, the 4-1BB/4-1BBL signaling leads to CD8 T cell survival, and forming a larger CD8 memory T cell pool[6].

Much evidence suggests that CTL responses are the key point of a successful vaccine against HIV[7], but early qualitative and late quantitative abnormalities in CD4 T cells are observed typically in HIV-1 infected individuals and the deficiency of CD4 Th function results in the inability of CD8 T cells[8]. In this paper we utilized both 4-1BBL and OX40L, which have been illustrated to be critical for effective CD8 and CD4 functions[9], as adjuvants for the multiple-epitope DNA vaccine against HIV. This is the first example of modulating the immune response to a HIV multiple-epitope DNA vaccine by the coexpression of dual costimulatory molecules. We hypothesized that direct targeting both CD8 T cells and CD4 T cells might be an effective strategy for designing new anti-HIV vaccine.

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2 Materials and Methods

2.1 Reagents

The fluorochrome-conjugated Abs directing against human CD4, CD8, CD86, CD83, HLA-DR and IFN-γ, as well as matched isotype Ab controls were obtained from eBioscience. IL-10, IL-4, and IL-2 were detected with the human Th1/Th2 cytometric ELISA assay kit(eBioscience). CTL assays were detected with non-radioactive cytotoxicity assay kit (Promega). The HIV HLA-A0201-resticted peptide sets(according to vaccine, purified to >90%) and p24 peptide sets(15 aa with 11-aa overlaps) were obtained from the HD biosciences. rhIL-2, rhIL-4, rhGM-CSF, rhTNF-α and rhIL-6 were purchased from PeproTech for cell culture.

2.2 Recombinant Plasmid

Recombinant plasmids were constructed as previously described[10]. Briefly, human 4-1BBL and OX40L genes were synthetized by Generay Biotech Co. Ltd., and were inserted into the downstream of the MEG-p24 gene(including HIV-1 p24 gene and highly conserved and immunogenic T-cell and B-cell antigen genes of pol, Rt, Vpr, Tat, p17, gp160 and nef) of the plasmid vector pVAX1-MEG-p24(pMp). By this means, dual costimulatory molecules DNA vaccine pVAX1-MEG-p24-4-1BBL-OX40L(pMpBO) and single costimulatory molecule DNA vaccine pVAX1-MEG-p24-OX40L(pMpO) were obtained.

Expression of the recombinant plasmids was confirmed using RT-PCR and fluorescent identification(data not shown).

2.3 Dendritic Cell Generation, Transfection and Phenotyping

All healthy donors were screened for HLA-A0201 by FACS analysis. DCs generation was harvested by leukapheresis. Briefly, freshly obtained PBMCs were plated at 3 million/well in a 24-well plate and left for 2 h to allow the monocytes to attach to the plastic wells. T cells(purified by the pan T cell-negative selection kit) were resuspended in RPMI 1640, in the presence of rhIL-2(800 U/mL) and then was plated at 5×10^5 cells/well in a 24-well plate. After washing the adherent cells, the CRPMI 1640 medium[11] containing rhGM-CSF(100 ng/mL) and rhIL-4(200 ng/mL) was added to the wells. Immature DCs(iDCs) collected on Day 3 were evaluated in functional assays, and were transfected with recombinant plasmids by square wave electroporation as described previously[12], and then cultured for 2 more days with 10 ng/mL rhIL-1, 10 ng/ml rhIL-6, 20 ng/mL rhTNF-α, and 500 ng/mL PGE2 for facilitating maturation. The following panel of FITC- and PE-conjugated antibodies was applied to flow cytometry phenotyping assays of mature DCs: anti-human CD86, HLA-DR, CD83. FITC and PE-conjugated IgG1 and IgG2b antibodies of irrelevant specificities were included as negative controls.

2.4 Flow Cytometry

For intracellular staining, cells were restimulated with 5 μmol/L peptide and incubated with Brefeldin A to facilitate intracellular cytokine accumulation, and after 16 h at 37 °C in 5% CO_2, the cells were washed with FACS buffer containing 0.1% azide and cells surface was stained with surface markers for 30 min at 4 °C. Cells were then fixed and permeabilised with the Cytofix/Cytoperm kit and stained with anti-cytokine Abs for 1 h at 4 °C, in all figures, the indicated gates were set based on staining with isotype control Ab for each sample. Cells were analyzed by flow cytometry using a FACSCalibur.

2.5 Measurement of Cytokines

Culture supernatants were harvested and stored at –80 °C until being used for analyzing secreted cytokines. IL-2, IL-4 and IL-10 production in the supernatants was assessed *via* the human Th1/Th2 cytometric ELISA assay kit.

2.6 Cytotoxicity Assays

For HLA-A0201 donors, T2 cells line was used as target cells in the CTL assay. T2 cells were pulsed with 5 μmol/L HIV peptide overnight, and then target cells and serial dilutions of effector cells in triplicate were incubated in complete RPMI 1640 medium for 4 h, and then cytoxicity assays were carried out with the non-radioactive cytotoxicity assay kit. Supernatants were detected for analyzing the release of lactate dehydrogenase(LDH).

2.7 Statistical Analysis

A two-tailed two-sample equal variance student's *t*-test was used for testing the significance of the data.

For the experiments of cytokine accumulation of cell cultures, data are presented as means±SDs.

3 Results

3.1 Phenotypic Characterization of DCs

After 48 h transfection with recombinant plasmids, the expressions of various membrane proteins located at the transfected DCs(tDCs) surface, including CD83, CD86 and HLA-DR, were first examined by flow cytometry(Fig.1). The expression of CD83 on DCs is generally assumed to indicate efficient APC-function, $CD83^+$ DCs on day 5 is mean about 88%(range 78%—94%), the percentages of $CD86^+$ and HLA-DR^+ cells were generally high, that of $CD86^+$ DCs on day 5 is mean about 95.8%(range 92%—97.6%) and that of HLA-DR^+ DCs is mean about 91.7% (range 84%—96%). Even the $CD83^-$ DC population was positive for CD86 and HLA-DR.

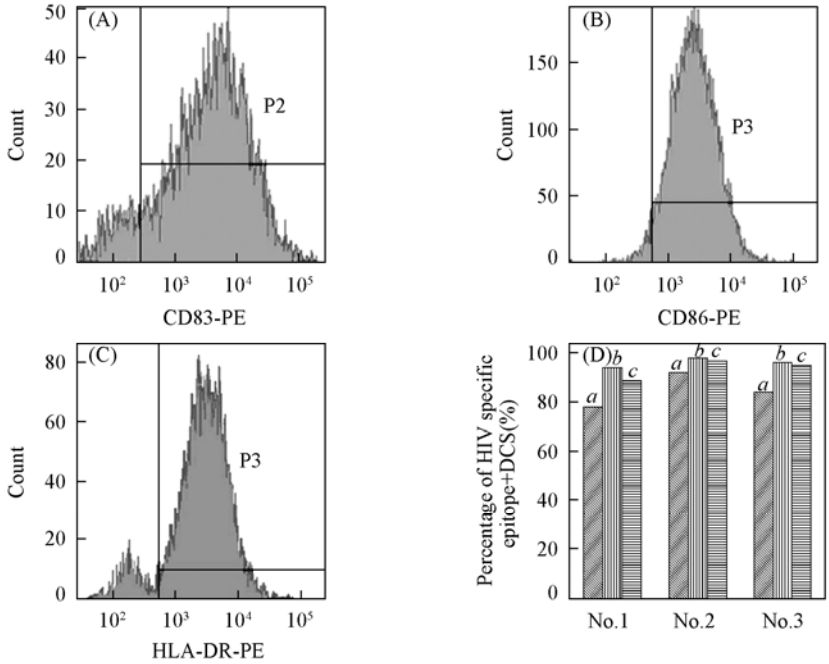

Fig.1 Phenotypic characterization of mDCs

(A)—(C) Representative data of the expression of cell surface markers CD83, CD86 and HLA-DR on day 5, respectively; (D) a summary of percentage of CD83(*a*), CD86(*b*) and HLA-DR(*c*) from other three donors.

3.2 Transfection Efficacy in Human DC Transfected with Plasmid DNA Using Electroporation Method

To evaluate the transfection efficacy, the surface expression of HIV specific epitopes on DCs at 48 h post transformation was checked by flow cytometry. The surface expression of HIV specific epitope was evident. Electroporation of plasmid DNA resulted in a mean transfection efficacy of 6.1%(Fig.2).

3.3 Effects of Dual Co-stimulatory Molecules on Cytokine Accumulation in Cultures

The overall levels of cytokines(IL-4, IL-10 and

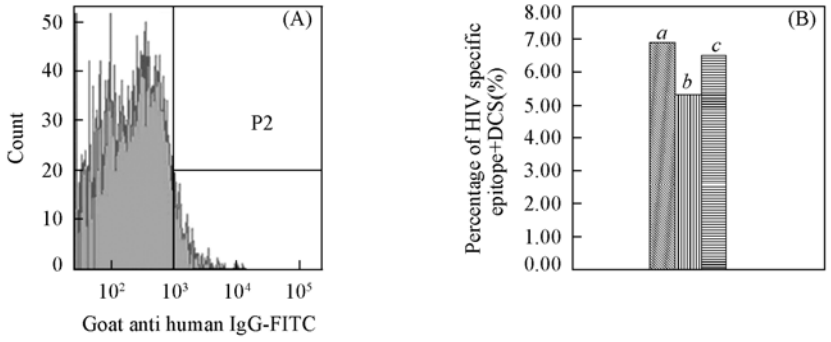

Fig.2 Transfection efficacy of human DC transfected with plasmid DNA *via* electroporation method

Plasmid DNA transfected mDCs were stained with HIV-1 positive serum Ab for 30 min, and then stained with FITC conjugated goat anti human IgG. (A) A representative experiment from four donors is shown, the transfection efficiency is 5.7%; (B) percentages of HIV specific epitope+ DCs from other three donors, the mean transfection efficiency is 6.1%. *a*. Donor No.1; *b*. donor No.2; *c*. donor No.3.

IL-2) from four healthy individuals were assayed (Fig.3). A facilitative effect of dual costimulatory molecules was observed as the accumulation of cytokines of IL-4 and IL-2. Both pMpBO and pMpO inhibited the secretion of IL-10, indicating that the coexpression of 4-1BBL and OX40L as adjuvant has more facilitative effects on cell-mediated immunity.

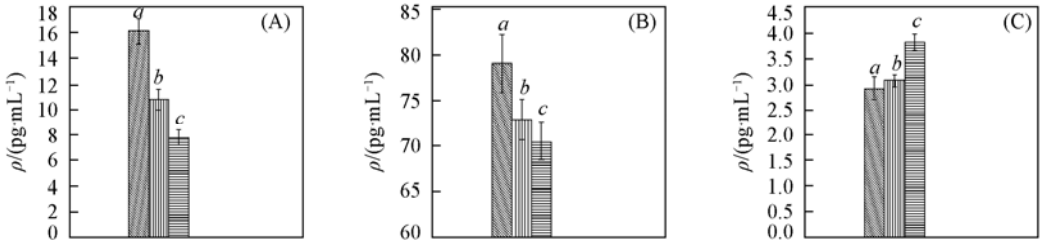

Fig.3 Cytokine accumulation in response to costimulation

Student's *t* test was used to determine statistical significance, with the *p* values indicated. Error bars indicate the SD in the experiment. (A) The accumulation of IL-2 in pMpBO(*a*) cultures shows the highest level in both donors, as compared with pMpO(*b*)($p<0.001$) or pMp(*c*)($p<0.001$); (B) the level of IL-4 in pMpBO(*a*) cultures is the highest, as compared with pMpO(*b*)($p<0.02$), or pMp(*c*)($p<0.005$); (C) the levels of IL-10 in both pMpBO(*a*) and pMpO(*b*) cultures are lower than that in pMp(*c*) cultures($p<0.001$), but there is no difference between pMpBO group and pMpO group($p>0.2$).

3.4 Effects on CD4 T Cell IFN-γ Production

tDCs and autologous T cells were cocultured in the absence of exogenous cytokines *in vitro* system, and re-stimulated in culture with the HIV peptide sets at the end of 7-day coculture. The production of IFN-γ in CD4 T cells was examined by intracellular cytokine Flow cytometry. A representative assay from donor No.4 is shown in Fig.4(A). tDCs transfected with pMpBO enhanced the ability of IFN-γ secretion of CD4 T cells, as compared with pMpO(increased by 2.07 folds, range from1.53- to 2.54-folds, $p<0.001$) or pMp(increased by 4.27-folds, range from 3.43- to 5.19-folds, $p<0.001$). A statistical analysis of IFN-γ production of CD4 T cells in donors Nos.1—3 is shown in Fig.4(B).

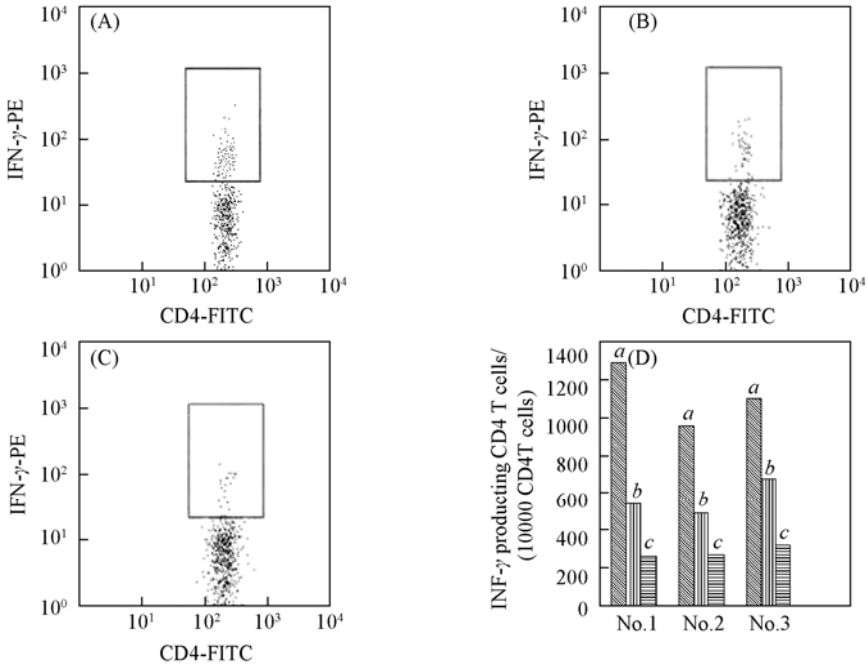

Fig.4 IFN-γ production by CD4 T cells in response to costimulation

(A) PMpBO; (B) pMPO; (C) pMP; (A)—(C) A representative assay from participant No.4. Cells were analyzed for CD4 and IFN-γ. Data shown are gated on CD4 events. The number indicates the percentage of IFN-γ-positive cells; (D) percentages of IFN-γ producting CD4 T cells per 10000 CD4 T cells from other three donors. *a*. pMpBO; *b*. pMpO; *c*. pMp.

3.5 Effects on CD8 T Cell Cytotoxicity

An *in vitro* cocultured system of tDCs and T cells in the absence of exogenous cytokines was used to expand CTL responses. A representative experiment from one donor is illustrated in Fig.5. Compared to either pMpO or pMp, pMpBO resulted in a dramatic improved CTL response. Each subject was tested in duplicate and consistent results were observed.

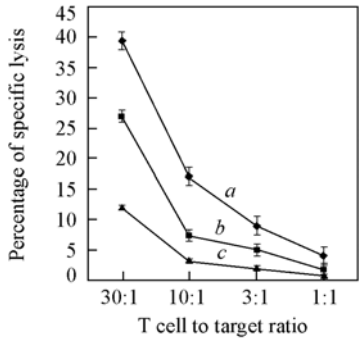

Fig.5 A representative percent specific lysis of HIV peptide-coated T2 target cells by T cells

a. pMpBO; *b*. pMpO; *c*. PMp. Similar results were obtained with other three donors, with experiments repeated twice per donor.

3.6 Effects on CD8 T Cell IFN-γ Production

The activity of CTL effector was assessed by intracellular IFN-γ production, as shown in Fig.6(A). tDCs transfected with pMpBO dramatically enhanced the ability of IFN-γ secretion of CD8 T cells, as compared with pMpO(increased by 2.38 folds, range from1.82- to 3.18-folds, $p<0.001$) or pMp(increased by 4.47-folds, range from 3.46- to 5.67-folds, $p<0.001$). A statistic analysis of IFN-γ production of CD8 T cells in No. 1—3 donors are shown in Fig.6(B).

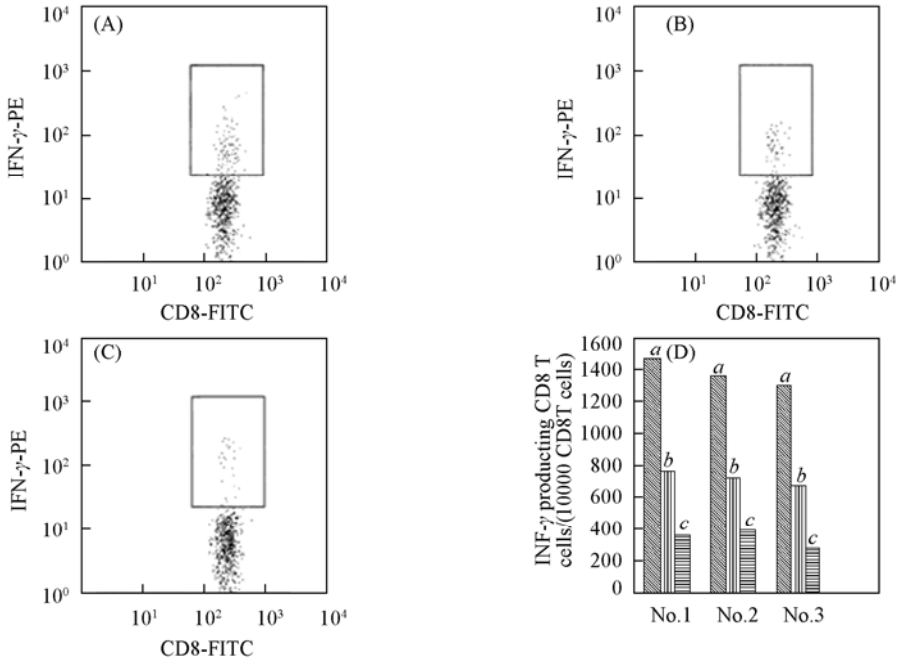

Fig.6 IFN-γ production by CD8 T cells in response to costimulation

(A) pMpBO; (B) pMpO; (C) pMp. Cytokine production was assessed by intracellular staining. In all figures, the indicated gates were set based on staining with isotype control Ab for each sample. (A—C) Representative data; (D) similar data obtained from other three donors. *a*. pMpBO; *b*. pMpO; *c*. pMp.

4 Discussion

Scientists have made great efforts to enhance HIV-specific CTL responses, including the usage of fowlpox[13] or MVA vaccine[14] or co-administration of cytokines with DNA vaccine[15]. Our group has constructed a recombinant DNA vaccine pVAX1-MEG-p24 and has been successful in applying this technique to mouse model and rhesus model[10]. In this study, two costimulatory molecules, 4-1BBL and OX40L, were coexpressed within the recombinant DNA vaccine pVAX1-MEG-p24 as adjuvants and this recombinant has been shown effective in modulating the anti-HIV immune response.

4-1BBL is a robust costimulation factor emerging as an important regulator of CD8 T cell response. Intracellular IFN-γ staining confirmed that 4-1BBL could effectively enhance CD8 T cell response when 4-1BBL was coexpressed within a recombinant fowlpox virus expressing HIV antigens in a poxvirus prime-boost vaccine strategy in a mouse model[16]. CD4 T cells are critical to the maintenance of an effective ongoing CD8 CTL response. Ligation of OX40 on CD4 T cells can dramatically enhance their ability to help virus-specific CTL responses against HIV-1 in human. Owing to greater OX40 expression in CD4 T cells in the HIV-1 infected individuals allowing more targets for OX40L, OX40L can make HIV-1 specific CTL responses from HIV-1 infected individuals greater than EBV-specific responses from uninfected individuals[17]. Thus, we hypothesized that the coexpression of 4-1BBL and OX40L as adjuvants in HIV DNA

vaccine may be of greater benefit to HIV infected individuals.

Transferring a genetic material into DCs is a very prospective tool for developing new vaccines in anti-HIV treatment. Based on human dendritic cell-T cell coculture system, total T cell culture was primarily studied, as we tried to mimic a similar therapeutic context, in which CD8 T cell responses to Ag plus costimulation can be induced in the presence of a full complement of T cells. We examined the modification of the transfected DCs(tDCs) surface expression levels of CD83, CD86 and HLA-DR on day 5(Fig.1). The expressions of CD86 and HLA-DR were increased by 95.8% and 91.7%, respectively, and the expression of maturation marker CD83 was increased by 88%. This indicates that the DCs became fully mature on day 5. Electroporation has been used to transfect plasmid DNA to monocytes derived DC. A drawback of this method is that low transfection levels may be produced. In this study, we observed a transfection rate of 6.1% for plasmid DNA(Fig.2).

We examined cytokine accumulation in cultures. When total T cells were cocultured with DNA vaccines transfected DCs, significant enhancement of IL-2 accumulation was detected *ex vivo* in the culture of pMpBO(Fig.3). Although IL-4 level in the culture of pMpBO is the highest in the three groups, IL-10 levels in the cultures of pMpBO and pMpO are lower than in that of pMp(Fig.3). So we supposed that dual co-stimulation molecules have more facilitative effects on cell-mediated immunity. Although 4-1BBL has been confirmed to have the ability to induce Th1 response[18], the controversy regarding the effect of OX40L on the Th1 or Th2 immune response is not solved yet[19]. The effect of dual co-stimulatory molecules DNA vaccine on human humoral immunity should be further examined *in vivo* experiments in the future.

In this study, we observed that coexpression of 4-1BBL and OX40L as adjuvants in HIV multiple-epitopes DNA vaccine resulted in the highest frequency of IFN-γ producing CD4 and CD8 T cells *in vitro* in human(p<0.001)(Figs.3 and 6). Some literatures suggested that an excessive accumulation of IFN-γ in cultures of total T cells from healthy donors resulted in the inhibition of further CD8 T cell expansion[20]. In contrast, we observed a significant enhancement of peptide-specific antiviral CD8 T cell responses to pMpBO(Fig.5). The data suggest that the dual co-stimulatory molecules can effectively increase the production of cytokine and the function of it in CD8 T cells.

Effective and sustained memory responses are important to the control of persistent viral infections. OX40L is important to maintain potent memory T cell responses[21] and other work has confirmed that 4-1BBL costimulation is an important regulator in enhancing CD8 T cell memory[6]. So the examination of the effect of the dual co-stimulatory molecules DNA vaccine on memory responses should be addressed in future studies.

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