Combined Transfection with EBV-Specific Epitopes and HLA-A2 genes is More Effective than Separate Transfection in Promoting CTL Lysis against Nasopharyngeal Carcinoma

Weijun Ding [1, 3] and Choylen Fong[2]

To augment specific cytotoxic T lymphocyte (CTL) lysis is a promising strategy for cancer therapy. In this study, we examined the boosting effect of CTLs upon autologous lymphoblastoid B cell lines (LCLs) transfected with diverse plasmids, to explore the possible CTL-based immunotherapy of nasopharyngeal carcinoma (NPC). FCM analysis displayed rather high ratio (>30%) of successfully transfected LCLs by utilizing the DMRIE-C kit. CTL assays demonstrated that substantially higher ratio of CTL specific lysis was observed upon the LCLs transfected with both expression vectors encoding EBV-specific epitopes and their presentation molecule HLA-A2, in contrast with those transfected separately. By transfecting the vector encoding HLA-A2 alone, only the LCLs of HLA-A2$^+$ donors elicited markedly higher CTL lysis. CTL assays also showed that there existed no marked differences upon transfection by either different vectors (pcDNA3, pNGVL3 or pNGVL3-hFlex), or different EBV-derived peptides (LMP_2Pep1 or LMP_2Pep2), or with or without the doubled DNA sequence encoding peptides. This study indicated a promising immunotherapy strategy on NPC through boosting and eliciting the EBV-specific CTL activation by transferring vectors encoding both EBV-specific epitopes and their presentation molecule HLA-A2 into autologous LCL, the presentation cells of MHC/peptide tetrameric complex. *Cellular & Molecular Immunology.* 2004;1(3):229-234.

Key Words: NPC, LMP_2, LCL, HLA-A2, CTL

Introduction

Based on statistic reports (1, 2), 98% of nasopharyngeal carcinoma (NPC) patients are Chinese, especially south-eastern Asian Chinese. It is even named after a southern Chinese province as Cantonese Cancer. Therefore, we can expect that genetic backgrounds might be one of the important factors upon NPC. Besides genetics, there are much complex etiological factors upon this cancer. Immunologic factors, reserved foods, over-salt, smoking, various infecting factors are also relevant with NPC (2, 3, 4). Now, Epstein-Barr virus is believed to be the most important biological factor of NPC (1, 2, 4, 5).

[1]Laboratory of Microbiology and Immunology, Chengdu University of Traditional Chinese Medicine, 37# Shier Qiao Street, Chengdu 610072, China.

[2]Laboratory of Immunotherapy, Division of Cellular and Molecular Research, National Cancer Centre of Singapore, 11 Hospital Drive 169610, Singapore.

[3]Corresponding to: Dr. Weijun Ding, Laboratory of Microbiology and Immunology, Chengdu University of Traditional Chinese Medicine, 37# Shier Qiao Street, Chengdu 610072, China. Fax: +86-28-87763471, Tel: +86-28-85414242, E-mail: dingwj123@163.com.

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Epstein-Barr virus (EBV) is the best known and most widely studied herpes virus, due to its clinical and oncogenetic importance. As long-term parasite and co-evolution with human being, EBV has co-evolved and been adapted as latent infection within the immune cells of the host (1, 3). Because EBV can elicit a strong HLA class I restricting CTL response in the majority of infected individuals, the frequency of EBV specific cytotoxic T lymphocyte precursors (CTLp) in the circulating T cell pool of health individuals is unusually high (at least 1 among 10,000 circular T cells) and also remarkably stable (1, 3, 6). So it is believed that the CTLs play an important role both in controlling the EBV from primary infection and long-term carrier state. About 99% of human population are EBV-carriers, but less than 0.1% of them elicit diseases such as NPC, Burkitt's Lymphoma, Hodgkin's disease and Polyclonal Lymphomas (1, 4). It has accumulated an immense body of information to confirm that during the long progress from the beginning through NPC formation, some kinds of abnormal EBV-based specific cytotoxic reaction should exist. Like other tumors, almost all of the NPC patients demonstrate quite lower EBV specific CTL reactions than those of health carriers. Therefore, many NPC researches have focused their studies on boosting EBV specific CTL. Currently, major methods employed are:

Abbreviations: CTL, cytotoxic T lymphocyte; EBV, Epstein-Barr virus; HLA-A2, human leukocyte antigen A2; LCL, lymphoblastoid B cell line; LMP_2, latent membrane protein 2; NPC, nasopharyngeal carcinoma; PBMC, peripheral blood mononuclear cell.

1) To increase the co-stimulators, such as the B7 family; 2) To boost the expression of relevant cytokines, such as IL-2, IL-4, TNF and IFN; 3) To stable the peptide-MHC complex; and 4) To enhance the expression of class I MHC/peptide tetrameric complex on the surface of antigen present cell or their target B lymphocytes (3, 7, 8).

Although relevant researches can be found easily, most of them merely study on one of the above aspects, either on the co-stimulators, antigen-presenting molecules or EBV-based epitopes. Such methods can hardly counteract the immunosuppression of NPC and its profound strategy of escaping from tumor immuno-surveillance (2, 3, 7, 8). In order to amend this abnormal low expression, and stable the MHC/peptide tetrameric complexes, for the first time we design this present strategy to increase both expression and presentation in activation of the EBV-based CTLs, by means of transfection of vectors encoding both EBV-derived epitope and the presentation molecule HLA-A2 into autologous lymphoblastoid B cell lines (LCLs). In this paper, we report the results of *in vitro* CTL assay.

Materials and Methods

Blood sample collection and HLA type assay

Blood samples of NPC patients were collected from National Cancer Center, Singapore. About 20ml of blood taken from each patient was fractionated on Ficoll density gradients. Peripheral blood mononuclear cells (PBMC) were harvested and divided into three aliquots. One was used for the establishment of EBV-transformed LCL by addition of exogenous EBV (B95.8 strain), another was HLA typed by means of FCM by utilizing the mouse-anti-human HLA-A2 antibody and the FITC-conjugated antibody (second antibody), and the rest crystal-preserved for further analysis.

Peptides and recombined plasmids

Two kinds of peptides representing MHC class I-restricted EBV epitopes were listed as follows: LMP_2Pep1, S-L-G-G-L-L-T-M-V; LMP_2Pep2, L-L-W-T-L-V-V-L-L. They were commercially synthesized and RP-HPLC purified by Alta Bioscience (University of Birmingham, Birmingham, U.K.), and the purity levels were 80% or higher. They were used as stimulator to induce CTL clones recognizing MHC class I-restricted EBV-specific epitopes.

Sequences decoded from the above EBV epitopes of LMP_2 Pep1 and LMP_2 Pep2 and the double of decode region sequence (LMP_2 Pep1 2× and LMP_2 Pep2 2×) were synthesized by GIBCOBRL, Life technologies. Besides decode region, they also contain the starting and stoping codes, and the EcoRI and BamHI site; which were listed as follow: LMP_2 Pep1, 5'- AAA GAA TTC ATG AGC CTG GGT GGC CTC CTA ACA ATG GTC TGA GGA TCC CC-3'; LMP_2 Pep1 2×, 5'- AAA GAATTC ATG AGC CTG GGT GGC CTC CTA ACA ATG GTC AGC CTG GGT GGC CTC CTA ACA ATG GTC TGA GGA TCC CC-3'; LMP_2 Pep2, 5'- AAA GAA TTC ATG CTG TTG TGG ACG CTG GTC GTG CTG CTC TGA GGA TCC CC-3'; LMP_2 Pep2 2×, 5'- AAA GAA TTC ATG CTG TTG TGG ACG CTG GTC GTG CTG CTC CTG TTG TGG ACG CTG GTC GTG CTG CTC TGA GGA TCC CC-3'.

Three kinds of plasmids were chosen for construction expression vectors. The pcDNA3 was used as the vector encoding HLA-A2, while pNGVL3 and pNGVL3-hFlex were the vectors encoding EBV special peptides. pNGVL3-hFlex contains a supplemental tail flt3 ligand, a cloned hematopoietic growth factor that markedly augments the number of active dendritic cells and NK cells in lymphoid and non-lymphoid tissues and exerts anti-tumor activity in various experimental models (9, 10). These recombined vectors were confirmed by enzyme digestion assay and DNA sequence employing the automated cycle sequence machine (ABI 377, Macintosh).

Generation of LCL by EBV transforming of B lymphocytes

As an established methods, Epstein-Barr virus infection of B lymphocyte *in vitro* gives rise to immortalized LCL (3, 6, 11). Briefly, take about 10^5 of PBMCs in 2.5 ml completed RPMI-1640 (containing 10% FCS, 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg of streptomycin) into a 50-ml conical tube, add 2.5 ml stored EBV-containing culture supernatant of B95.8, and incubate cells in 37 ^oC water bath for 2 h. Then add 5 ml completed RPMI-1640 which contains 1 μg/ml cyclosporin A for inhibiting the growth of T lymphocytes, and transfer to 25-cm^2 tissue culture flask. Stand flask for 3 weeks in 5% CO_2 incubator, and the completed LCL could be obtained. Mix cells and divide them into two new flasks, add 5 ml completed RPMI-1640 and incubate cells for 1 week, and then maintain cell line by splitting 1:3 in completed RPMI-1640 once a week.

Generation of CTL clones

Special CTL clones for recognizing certain EBV-based MHC class I restricted epitopes were established as below. Thaw the stored PBMCs (2×10^6) and put into 24-well plate, incubate with 50 μM of the peptide in 100 μl of RPMI-1640 for 2 h at 37 ^oC. Then wash and re-suspend in 2 ml completed RPMI-1640 medium supplemented with 25 ng/ml IL-7 (Sigma). After 3d, add IL-2 (10 U/ml, Sigma) to the medium. Thereafter, culture was refreshed twice weekly. From day 12 forward, re-stimulate weekly with irradiated (4000rad) autologous LCL (10^5/ml) and pulse with 10 μM peptide. After day 14, test the monoclonal CTL cultures weekly by two ways. One test was observation under the invert phase contrast microscope to score the CTL clones, which could be distinguished by their larger or some irregular shape and bright appearance; another way was cytotoxicity assay by using the CTL sensitive target cell line K562. Once established, maintain the special T cell clone in T cell growth medium containing rIL-2 20 U/ml, amplify culture by utilizing the completed RPMI-1640 medium added with rIL-2 (20 U/ml) and autologous LCL (10^5/ml), then store at -130 ^oC.

Cytotoxic T lymphocyte assay

CTL Clones derived from NPC patients were screened for EBV specificity using a standard 5h chromium release assay (6). Target cells, the autologous LCL, were infected with recombinant vectors encoding EBV special epitopes or/and the HLA-A2 for 2 h at a multiplicity of infection of 10, followed by 16 h of incubation in growth medium. For effectively transfecting the suspension target cells, we utilized the special kit, DMRIE-C (Life Technologies, GIBCOBRL). Targets were washed, countered and adjusted into 2 $\times 10^5$/ml in completed medium, then labeled with ^{51}Cr and used in the assay. On the other hand, the effective cells, cytotoxic T cells, were adjusted into 2 $\times$ 10^7 /ml, and added into the 96-well plate at the E:T ratio 50:1, 25:1, 12.5:1, 6.25:1. Mix gently for 1 min at 500 rpm, incubate at 37 ^{o}C for 5 h, centrifuge plated for 5 min at 500 rpm, harvest supernatant to ^{51}Cr count tube and counter. All specific assays were repeated at least three times (11, 12, 13).

Results

General conditions of the collected blood samples from NPC patients

Five blood samples were taken from the NPC patients in the affiliated hospital of National Cancer Centre (NCC), Singapore. All of the five patients were Chinese, 2 females and 3 males. They had a mean age of 45 ± 6.2 years (range, 37-55). According to the authoritative standard issued by UICC and AJCC, clinic NPC stages of these patients were identified as IIA, IIB or III, respectively (Table 1). The PBMCs of 8 to 20 million were obtained from each sample. Results of HLA type showed that there were three HLA-A2 positive (NPC No.2, 4 and 5) and two negative (NPC NO.1 and 3). Five LCLs were obtained by utilizing the supernatant of B95.8 culture to transform the PBMCs. The successfully transformed LCLs had a "hand-mirror" shape and often grew in large clumps (1, 12), and were confirmed by FCM assay (antibody CD19). Monoclone T cytotoxic cells were obtained from PBMCs pulsed by both EBV-specific peptide and autologous irradiated LCL.

The DMRIE-C kit displayed rather high ratios of successfully transfected LCL

Table 1. *General conditions of NPC patients providing blood samples in this study.*

NO.	Age	Sex	HLA-A2 Type	Clinic stage*	Ethnic group
1	37	Male	-	IIA	Chinese
2	43	Female	+	IIB	Chinese
3	55	Male	-	IIB	Chinese
4	46	Male	+	III	Chinese
5	39	Female	+	IIB	Chinese

*According to the international standard of NPC stage issued by UICC&AJCC (1997, 5th version).

Compared with the transfection of adhesive cell lines, suspended cell lines such as our LCL are always more difficult to be transfected. So we employed a special kit, the DMRIE-C especially designed for suspended cell lines, to conduct our experiments. After optimizing the protocol of transfecting the suspended cell line LCL, rather high ratios of successfully transfected LCL were observed by FCM (Figure 1). In this figure, the negative control that transfected by avoid vector pCDNA3 showed scarcely visible HLA-A2-LMP$_2$Pep1 complex in the cell surface. While in those transfected by pCDNA3-HLA-A2 + pNGVL3-LMP$_2$Pep1, pNGVL3-LMP$_2$Pep1 and pCDNA3-HLA-A2, respectively, the relevant data were markedly promoted. Such ratios of transfection were suitable for further CTL assays.

Striking higher CTL lysis were demonstrated in HLA-A2$^+$ donors than those of negative donors

First, we noticed that there existed significant differences of CTL assay results between the HLA-A2 positive and negative donors (Figure 2). After transfecting the target autologous LCLs derived from HLA-A2$^+$ patients (i.e. No.2, 4 and 5 patients) by various combinations of vectors, markedly higher ratios of EBV-specific CTL lysis were executed. On the contrary, similar CTL assays based on the LCL derived from HLA-A2$^-$ donors (i.e. NO.1 and 3 patients) were performed, but there were no likely promotion of CTL lysis. Such results confirmed one of the

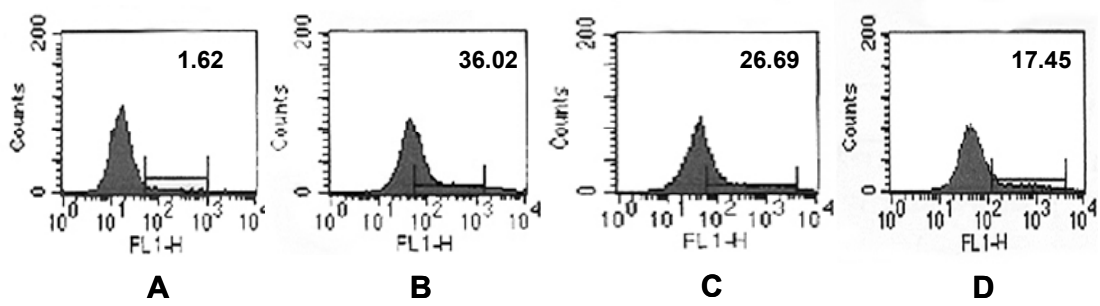

Figure 1. FCM showed rather higher ratios of successfully transfected LCL. A special kit, the DMRIE-C especially designed for suspended cell lines, was employed to conduct the transfection. The HLA-A2-LMP$_2$Pep1 complex was stained on the surface of target LCL from HLA-A2$^+$ donor for displaying the expression intensity of each test. It showed scarce HLA-A2-LMP$_2$Pep1 complex on LCL derived from HLA-A2$^+$ donor transfected by avoid vector pCDNA3 (A). The results also indicated the different expression of the same complex on the LCL derived from the same donor but transfected by pCDNA3-HLA-A2+pNGVL3 -LMP$_2$Pep1 (B), pCDNA3-HLA-A2 (C), or pNGVL3 -LMP$_2$Pep1 (D), respectively.

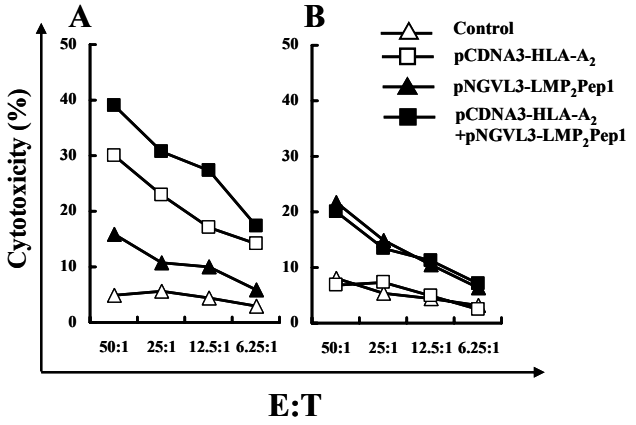

Figure 2. Significant higher ratios of CTL lysis were observed on target LCLs derived from HLA-A2$^+$ donors than those from negative donors. The ordinate values in each graph indicate the ratios of special CTL lysis (%). The control indicates a void vector transfection. Effectors utilized in each CTL assay were pulsed by LMP_2Pep1. (A) indicates the mean of the data derived from the HLA-A2$^+$ donors (NO.2, NO.4 and NO.5), respectively; (B) indicates the mean of the data based on HLA-A2$^-$ donors (NO.1 and NO.3).

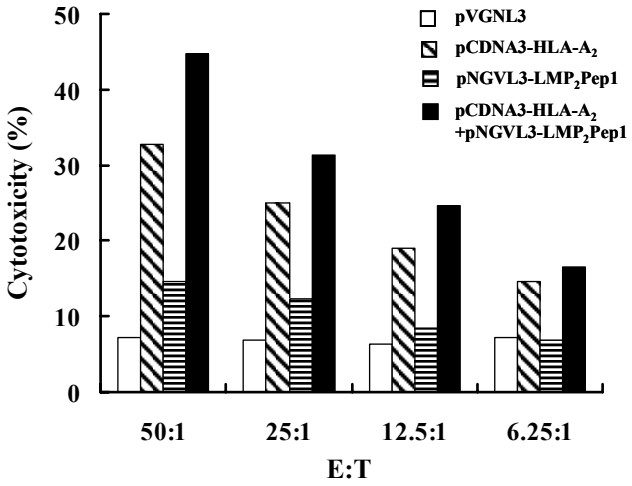

Figure 3. The combined transfection of both EBV-based epitope LMP_2Pep1 and the presentation molecular HLA-A2 induced substantially higher ratios of CTL lyses than those separately transfection. The ordinate values indicated the ratios of special CTL lyses (%),which were the arithmetic average of the CTL assays based on the three LCLs derived from HLA-A2$^+$ donors (i.e. NO.2, NO.4 and NO.5). By transfection of HLA-A2 alone could induce mild higher CTL lyses. And by transfection of EBV-specific LMP_2 Pep1 alone, only slightly higher CTL lyses were displayed, compared with those LCL transfected by void plasmid.

principal rules that CTL recognized both MHC molecular and linear peptide sequence that lies along the groove of MHC outermost domain.

Substantially higher ratio of CTL lysis was observed on LCL transfected with expression vectors encoding both EBV-specific epitopes and their presentation molecular HLA-A2

The main topic of this article is to discover whether combined transfection of EBV-based epitopes and their presentation molecular HLA-A2 is more effective in promoting CTL lysis than separate transfection. Figure 3 listed one of the most interesting results in this study. From this figure we can see that the LCL from the HLA-A2$^+$ NPC patient (i.e. No.5 donor) were transfected by both pNGVL3-LMP_2Pep1 and pCDNA3-HLA-A2, and displayed the highest CTL effect, in contrast to those of LCL separately transfected by either pNGVL3-LMP_2Pep1 or pCDNA3-HLA-A2, or by void vector pNGVL3. Therefore, combined transfection of the target autologous LCL would conduct substantially higher CTL lysis ratios than those of LCLs transfected by either EBV-based epitopes or HLA-A2. Such finding may imply a new gene therapy strategy for NPC.

There also existed some interesting clues for our further study about the treatment of NPC

Firstly, as shown in Figure 4, in our experimental system *in vitro*, the utilization of expression vector that has an additional flt3 ligand did not display significantly higher ratios of CTL lysis than those without it. Although several experiments showed slightly higher lysis ratio, almost all of the CTL assays could not show distinguish promoting of CTL activation after adding flt3 ligand.

Secondly, the transfection experiments by employing the vectors that contained a doubled encoding region for EBV specific epitope (e.g. pNGVL3-LMP_2Pep1 2×) did not display markedly higher CTL lysis than those not doubled. Even though some experiments showed a mild higher, we still accept a general conclusion that the doubled DNA sequence encoding EBV-special epitope can not improve the presentation of HLA-A2-LMP_2Pep1 complex in the cell membrane of LCL, and can not promote substantially higher CTL lysis.

Finally, transfection by vectors encoding different EBV-special epitopes (i.e. LMP_2Pep1 and LMP_2Pep2) also did not manifest significant differences within our limited CTL assays. As a principal rule, the epitope is one of the key factors to affect the intensification of CTL lysis. Therefore so many studies have been accumulated upon exploring the most effective epitope for anti-caner treatment.

Discussion

It is known to all that the transfection of genes encoding tumor specific antigens or epitopes into antigen presenting cells to improve special CTL activation is a promising approach to develop cancer vaccines. Accordingly, lots of immunotherapy researches of NPC were focused on enhancing EBV special CTL activation (3, 7, 8, 15, 16). Since in NPC, EBNA1, EBNA3, LMP_1 and LMP_2 are the only virus proteins (15, 16), and endogenously expressed EBNA1 is protected from CTL recognition; consequently, LMP_1 and LMP_2 are the most likely target antigens for anti-tumor immunotherapy (15, 17). Furthermore, LMP2 epitope sequences (in contrast to EBNA 3A, 3B, and 3C and LMP_1 epitope) were shown to be most antigenically conserved among EBV (1, 13, 15).Therefore, LMP2 is

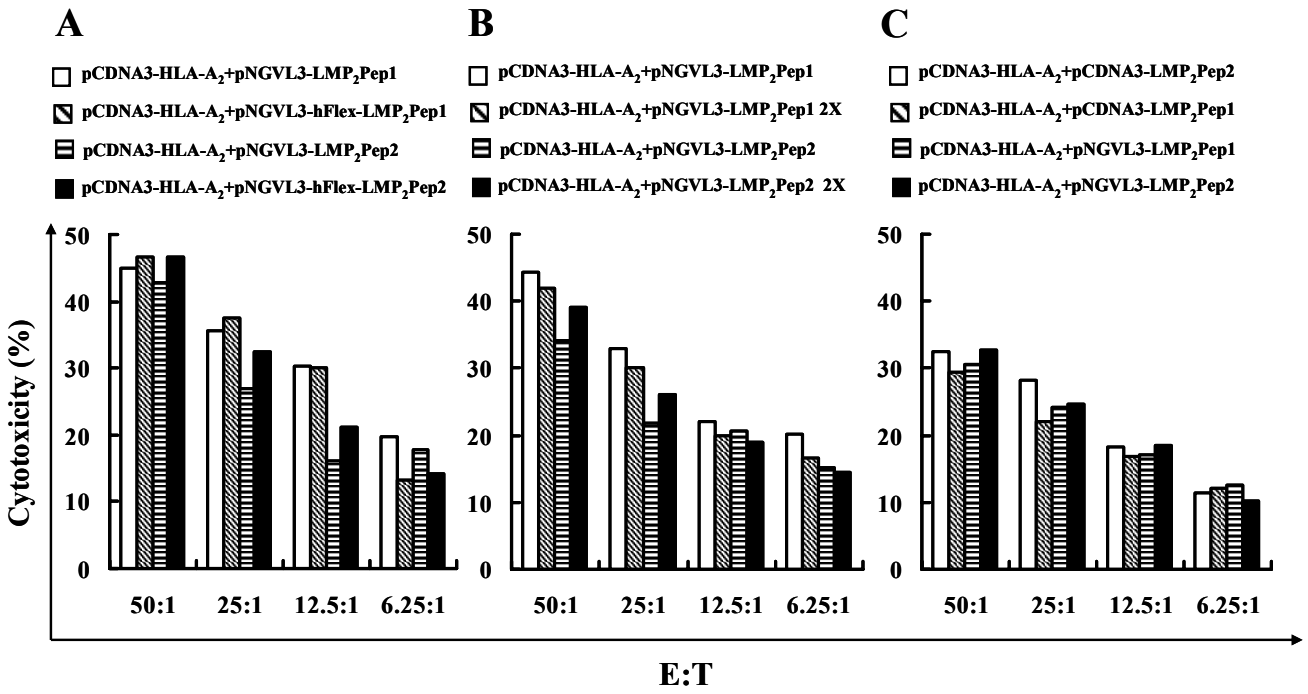

Figure 4. Other findings from our series of CTL assays may contain some interesting implies. The three kinds of CTL assays were designed to test the impact of hFlex, doubled encoding sequence or different EBV-derived peptides (LMP_2pep1 or LMP_2Pep2). The target cells were LCLs transfected by various plasmids marked in each graph, and effector cells were their relative CTLs. Although all of these factors were evidenced formerly as promoting elements to special anti-tumor activity, in these series of CTL assays there had no marked impacts.

more important than LMP1 in CTL response in a long-term of chronic infection. Here we focused on transfection genes encoding epitopes derived from LMP2.

On the other hand, it is also common knowledge that one of the most significant characteristics of cancer cells is the marked down-expression of all kinds of HLA molecular, which might be the most important mechanism of cancer cells to escape immuno-surveillance. As consequence, a number of gene therapy experiments based on promoting the expression of HLA can be found easily.

Obviously it is equally important to transfection by both EBV-specific epitope and the presentation molecular HLA, in order to upgrade the presentation of MHC/peptide tetrameric complex in the surface of target cells. But quite regretfully, almost all of the former studies were based on either EBV-specific epitope or HLA, none of the combined transfection. So this paper proposes a new approach to combine the two important factors in order to induce higher CTL lysis.

The combined transfection of vectors encoding EBV-special epitopes and their presentation molecular HLA-A2 demonstrated markedly higher CTL activation than those transfected separately. For example, to transfect LCL from the fifth donor by pCDNA3-HLA-A2 only, the CTL lysis ratio was 26.5 ± 3.22% (E:T=50:1); transfection of the vectors encoding EBV-specific epitopes induced 16.1 ± 2.5% lysis ratio; while transfecting these cells with the combined vectors, the lysis ratio were statistically higher (39.9 ± 2.2%). Similar results were observed in LCL from the other HLA-A2 positive donors. FCM and Western blotting reconfirmed the upexpression and presentation progress of MHC/peptide tetrameric complexes (data not shown). Principally, successfully transfecting them together means greatly increasing both components of MHC/peptide tetrameric complex presented in the cell membrane, and consequently strikingly promoting the CTL lysis. These results suggest a new immunotherapy strategy on NPC (1, 3, 16, 17).

Results of CTL assays also demonstrated that transfection of HLA-A2 could only boost the CTL lysis on LCLs derived from HLA-$A2^+$ donors. For the three HLA-A2 positive targets which were transfected by various combination of expression vectors, the transfection of PCDNA3-HLA-A2 displayed statistical significances of CTL lysis compared with the void vector. While in the HLA-$A2^-$ NPC patients, similar transfection showed almost no effect. The upexpression of MHC/peptide tetrameric complex were also observed in LCL derived from HLA-$A2^-$ donors (data not shown), but cannot be recognized by their T lymphocytes. Such results reconfirmed that CTL recognized both MHC and linear peptide sequence that lies along the groove of MHC outermost domains. Hence the HLA class I restriction is still a troublesome barrier on universe CTL-based therapy in clinics (1, 16, 17, 18). Very luckily, HLA-$A2^+$ is the most frequent MHC molecular in Chinese. Savage et al. found that 55.3% of Chinese in Singapore are HLA-$A2^+$. Thus, it may be suitable to choose HLA-$A2^+$ in prelude research on NPC therapy.

Other results in this article might bring us some useful suggestions. Firstly, although there are several *in vivo* experiments confirming that the supplemental tail flt3

ligand can markedly augment CTL lysis on cancer cells, in the present *in vitro* experiments it cannot. The flt3 ligand, as a cloned hematopoietic growth factor, executes its anti-cancer mechanism largely on boosting the functional dendritic cells and NK cells in lymphoid and non-lymphoid tissues (9, 10). Therefore it can be expected that *in vitro* CTL assay cannot see its promoting effect on special CTL function. Secondly, to assure the expression of EBV-specific peptides, we double their encoding DNA sequence within the vectors. But results showed that there existed no differences before and after doubled. These preliminary results imply that the doubled DNA sequence might not be necessary. Then, in order to find which vector is most effective for our transfection, we used three kinds of vectors. However, the results showed no difference of pNGVL3, pNGVL3–hFlex and pCDNA3, in contrast with relevant studies that showed somewhat different efficiency of transfection (11, 12, 14). It should be mentioned that our tests were conducted *in vitro* by employing the DMRIE-C kit, so further researches are necessary to find a proper and practical vector used *in vivo*. Finally, as for the two epitopes of LMP_2, no significant difference of special CTL lysis ratios was observed in our examinations, albeit there exit some differences in their chemical characters and stimulation effect on EBV-based CTL assays (15, 17, 18). As previously reported (1, 3, 14), the LMP_2 is more important than LMP_1 on the EBV-based CTL lysis in chronic malignancies. In fact, our selection of EBV special epitopes was based on these updated studies. As for exploring the most useful epitope for gene therapy of NPC, such important goal will entice further researches.

Taken together, this paper reports that transfection of vectors encoding both EBV-specific epitopes and the presentation molecular HLA-A2 into the autologous LCL can boost markedly higher CTL lysis than those of separate transfection. The CTL promoting effect was HLA class I-restricted. This approach of CTL-based therapy could be a promising strategy to malignancies such as NPC.

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