

Prime-Boost Immunization of Codon Optimized HIV-1 CRF01_AE Gag in BCG with Recombinant Vaccinia Virus Elicits MHC Class I and II Immune Responses in Mice

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The HIV-1 CRF01_AE gag gene was modified by codon restriction for *Mycobacterium* spp. and transformed into BCG; and it was designated as rBCG/codon optimized gagE. This produced 11 fold higher HIV-1 gag protein expression than the recombinant native gene rBCG/HIV-1gagE. In mice, CTL activity could be induced either by a single immunization of the codon optimized construct or by using it as a priming antigen in the prime-boost modality with recombinant Vaccinia virus expressing native HIV-1 gag. Specific secreted cytokine responses were also investigated. Only when rBCG gag was codon optimized did the prime-boost immunization produce significantly enhanced IFN- γ and IL-2 secretion indicating recognition via CD4+ and CD8+ T cells, and these responses seemed to be codon optimized immunogen dose-responsive. On contrary, the prime-boost vaccination using an equal amount of native rBCG/HIV-1gagE instead, or a single rBCG/codon optimized gagE immunization, had no similar effect on the cytokine secretion. These findings suggest that the use of recombinant codon BCG construct with recombinant Vaccinia virus encoding CRF01_AE gag as the prime-boost HIV vaccine candidate, will induce CD4+ Th1 and CD8+ T cell cytokine secretions in addition to enhancing CD8+ CTL response.

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INTRODUCTION

The usefulness of HIV-1 gag immunogens in vaccine development is supported by the fact that the protein is relatively conserved among different clades, and there is broad cross-clade CTL recognition (Clerici et al., 1996; Betts et al., 1997; Bertoletti et al., 1998; Durali et al., 1998). A number of various vaccine modalities have undergone preclinical and some clinical testing, including prime-boost strategies involving either DNA vaccines, proteins, live bacterial or viral vectors, with different degrees of immune responsiveness (Li et al., 1993; Hanke et al., 1998; Miyahara et al., 1998; Gonzalo et al., 1999). Live vaccines have been widely known as effective means of inducing robust cellular immune responses (Seder et al., 2000). Recently, we have shown that the prime-boost immunization comprising live recombinant *Mycobacterium bovis* BCG and Vaccinia virus substrain Dairen I (DIs) which has long been modified by attenuation in 1-day egg of the parent DIE strain (Tagaya et al., 1961; Kitamura et al., 1967) expressing HIV-1 CRF01_AE gag elicited cytotoxic T-lymphocytes more efficiently than that by either single vector alone (Promkhatkaew et al., 2009a).

However, direct cloning of target genes into heterologous expression systems often leads to suboptimal expression due to codon bias and may fail to stimulate strong immune responses. Interestingly, codon optimization of foreign genes compliant to host vectors or cellular systems has been shown to increase protein production resulting in enhanced immunogenicity. DNA vaccines with codon optimized HIV-1 gp120 significantly increased antibody titers and CTL activity (Andre et al., 1998), and tat induced superior T-cell proliferation, ELISPOT, and CTL responses (Ramakrishna et al., 2004), in mice. Moreover, mice and monkeys immunized with DNA containing modified gag gene showed quantitatively higher anti-gag Ab, CTL, and IFN- γ , than those immunized with a similar construct containing the native gene (Megede et al., 2000; Deml et al., 2001). Codon modified gag has also been used to induce higher frequencies of CD8+ CTL and circumvent pre-existing anti-viral vector immunity when the prime-boost regimens of DNA vaccine with adenovirus (Casimiro et al., 2003) and of only sequential adenovirus vectors of different strains have been performed (Pinto et al., 2003).

In some studies, antigen-specific CD4+ T-cell responses were observed consistently with those of CD8+ (Casimiro et al., 2003). It has been reported that codon-optimized genes induced a predominantly Th1 profile while the wild-type genes promoted a mixed Th1-Th2 type cytokine profile (Ramakrishna et al., 2004). Since Th1 cells express Th1-type cytokines, especially IFN- γ , and may also help in the differentiation and maturation of CTL,

we focused on IFN- γ and other cytokine related to the vaccine immunization. Consistent with the advantages of codon usage, in the present study we analyzed the impact of the antigenic protein expression in mycobacterial optimized HIV-1 gag as the priming candidate with recombinant Vaccinia virus containing native gene, on specific immune response parameters.

MATERIALS AND METHODS

Constructions of Recombinant BCG and Recombinant Vaccinia Virus

A whole gag DNA fragment of an HIV-1CRF01_AE isolate (GenBank Acc. No. AY863146) was amplified with primers having MunI or BamHI restriction sites at the 5'-end as 5'-ATATATCAATTGATCTAGCGGAGGCTAGAAGGAGAGAG-3', and 5'-ATATA TGGATCCCTAATACTGTATCATCTGCTCCTGTAT-3', respectively. The amplified DNA was then digested with MunI and BamHI and subsequently inserted into the respective endonuclease sites of pUC19 which already had been inserted with the hsp60 promoter downstream from the SacI site to introduce MunI before the multi-cloning sites and ampicillin resistance gene of the vector.

Following addition of KpnI linker at the EcoRI site of the previous plasmid, it was then cut with KpnI and ligated with the pSO246 plasmid (Matsumoto et al., 1996) harboring the mycobacterial origin of replication and kanamycin resistance genes. This plasmid was then transformed into the BCG strain Tokyo 72. This transformed BCG (rBCG/HIV-1gagE) was cultured by plating into the 7H10 agar containing 1.9% Middlebrook 7H10 (Difco, USA), 0.5% glycerol, 10% Middlebrook OADC serum (Difco, USA), and 10 μ g/ml kanamycin, for 3-4 weeks until colonies appeared. The culture was enriched by transferring a colony into the broth containing 0.47% Middlebrook 7H9 (Difco, USA), 0.5% glycerol, 10% Middlebrook ADC serum (Difco, USA) with kanamycin for 2-3 weeks. The pSO246 plasmid without insertion of respective DNA was also cloned into BCG as a recombinant BCG control.

To construct a recombinant Vaccinia virus expressing the same protein, the respective whole gag DNA was prepared as described earlier and refilled with nucleotides to be blunt-ended and inserted into the SmaI site of a plasmid containing Vaccinia virus early/late promoter p7.5, which had already been modified the HindIII site at the position of EcoRI site (Ishii et al., 2002). To obtain an appropriate plasmid for transfection into the Vaccinia virus DIs, the plasmid pUC/DIs was generated by joining a 1.9 kbp of specific genes deleted Vaccinia-DIs fragment into the pUC vector (Ishii et al., 2002). Finally, the fragment of gag adjacent to the p7.5 promoter as generated by digesting the previous recombinant Vaccinia virus DNA with HindIII was inserted into pUC/DIs at the same site. This recombinant plasmid was then transfected

into the parental recombinant Vaccinia-DIs virus during infection into primary chicken embryo fibroblast cells (CEF); this recombinant Vaccinia virus was designated as rDIs/HIV-1gagE. For the control virus, only pUC/DIs was transfected into the parental Vaccinia-DIs virus, which was designated as DIs/LacZ. The virus was cultured in CEF and determined the titer as described elsewhere (Ishii et al., 2002).

Construction of rBCG/Codon Optimized gagE

The whole HIV-1 CRF01_AE gag gene was designed and synthesized having the amino acid sequence described in GenBank Acc. No. AY863146 respective to the codons of *Mycobacterium* spp. according to the information described in the website *kazusa.or.jp*. Additions of nucleotides compliant with MunI and BamHI before the initiation codon and following the stop codon of the sequence respectively, were achieved. Then this codon optimized gag DNA was amplified and cloned into the promoter and origin of replication-modified vectors, and subsequently transformed into BCG as described earlier.

Western Blot for the Proteins Expressed from rBCG/HIV-1gagE, rDIs/HIV-1gagE and rBCG/Codon Optimized gagE

The rBCG/HIV-1gagE or rBCG/codon optimized gagE cell lysate, which was prepared by sonication, was run electrophoretically on 8%–20% gradient polyacrylamide gel and subjected to Western blot analysis utilizing the anti-HIV-1 gag p24 epitope monoclonal antibody (mAb) kindly provided by Professor K. Ikuta, the Research Institute for Microbial Diseases, Osaka University, Japan (Matsuo et al., 1992). The cell lysate of CEF cells infected with rDIs/HIV-1gagE was prepared for Western blot analysis as previously described (Ishii et al., 2002), and immunostained with the same anti-HIV-1gag p24 mAb.

Quantitative Determination of HIV-1 gag Proteins Expressed by ELISA

Five ml each rBCG/HIV-1gagE and rBCG/codon optimized gagE cultures in 7H9 broth was centrifuged and cell pellet was then resuspended in 200 μ l phosphate-buffered saline (PBS). The cell suspension was sonicated to collect cell lysate to be determined the amount of HIV-1 gag protein expressed according to the method described in the HIV-1 p24 antigen ELISA kit (Biomerieux, the Netherlands). To evaluate the gag protein expressed quantitatively, a standard HIV-1 p24 protein (United States Biological, Swampscott, Massachusetts, USA) was prepared in various concentrations and tested using the kit. The standard curve of p24 concentrations versus absorbances was used accordingly. Seven continuous sub-cultures of rBCG/HIV-1gagE and rBCG/codon optimized gagE were also done to determine the amounts of gag produced in comparison.

Mouse Immunization

First, 0.1 mg ($\sim 2 \times 10^6$ colony forming unit) or less of rBCG/codon optimized gagE was injected individually into BALB/c mice (H-2d strain) subcutaneously (s.c) between shoulder blades once, as a single immunogen or as a priming antigen prior to the first injection of the vaccinia-vectored rDIs/HIV-1gagE. The rDIs/HIV-1gagE virus grown in CEF cells was purified by centrifugation through 36% sucrose at 13,000 RPM for 90 min, resuspended in PBS, and the virus titer was determined.

Mice received 1×10^6 plaque forming units of rDIs/HIV-1gagE, injected intradermally (i.d) between shoulder blades, twice, 30 and 60 days after the priming injection of rBCG/codon optimized gagE or rBCG/HIV-1gagE. The mice were then sacrificed one month or longer after a single or the last immunization as summarized in Table 1. Control groups of mice were injected with the same amount of either recombinant BCG harbouring only the pSO246 vector (rBCG/pSO246) alone or with Vaccinia virus DIs harbouring the E.coli LacZ DNA vector (rDIs/LacZ). All animal experimentations were conducted under IRB of the institution and followed the guidelines of Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

Cytotoxic T-lymphocyte (CTL) Assay

In every assay, at least ten mice were used for each test group, and duplicate groups were done for each experiment. After immunizing BALB/c mice with an individual immunogen or prime-boost immunization, immunized or control mouse spleens were harvested and prepared as isolated spleen cells. To perform as effector cells, all spleen cells prepared from the group of 10 mice or more of the same immunization protocol were pooled and 10^7 cells were incubated for 5 days with different HIV-1 CRF01_AE gag peptide mixtures. Each mixture comprised five overlapping serial 20mer-peptides spanning 50–60 amino acids length. These resulted in 10 mixtures to cover the whole sequence of HIV-1 gag protein (498 amino acids), which represented serially amino acid positions 1–60, 51–110, 101–160, 151–210, 201–260, 251–310, 301–360, 350–408, 399–456, and 449–498.

Table 1: Mouse immunization and sacrifice schedule.

Regimen	Single or prime vaccine and schedule	Boost vaccine and schedule	Sacrifice schedule
Single immunization	rBCG/HIV-1gagE, Day 0	–	Day 70
Prime-boost immunization	1. rBCG/HIV-1gagE, Day 0	- rDIs/HIV-1 gagE, Day 30 and Day 60	Day 90
	2. rBCG/codon optimized gagE Day 0	- rDIs/HIV-1 gagE, Day 30 and Day 60	Day 90

For the target cells, 10^7 P815 mouse (H-2d strain) cells were infected with rDIs/HIV-1gagE for 18 h and labeled with 100 Ci $\text{Na}^{51}\text{CrO}_4$ for 90 min at 37°C , 5% CO_2 . The cells were then washed and resuspended as 10^5 cells/ml in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS).

To do the assay, 5×10^5 effector cells were placed into U-bottom microtiter plate, and 5×10^3 target cells were added into each well to yield 100:1 effector:target ratio, and incubated for 5 h. Spontaneous- and Total- ^{51}Cr release was estimated by keeping target cells with either the medium or 5% Triton X-100, respectively. The percentage specific cell lysis was calculated from the radioactive counts as $(\text{Sample release} - \text{Spontaneous release}) / (\text{Total release} - \text{Spontaneous release}) \times 100$.

IFN- γ Secretion Assay

First, 10^6 spleen cells from immunized mice were incubated 16 h with either the mixture of commercial clade B HIV-1 p17 and p24 proteins (United States Biological, Swampscott, Massachusetts, USA) 10 $\mu\text{g}/\text{ml}$ each, or 10 $\mu\text{g}/\text{ml}$ homologous recombinant HIV-1 CRF01_AE gag protein expressed by the intermediate recombinant pSO246/HIV-1gagE plasmid in *E. coli*, in RPMI-1640 medium containing 5% normal mouse serum at 37°C . (To obtain the recombinant protein, bacterial cells were harvested, lysed, and purified for gag protein by sucrose gradient ultracentrifugation, and the gag protein was then quantitated by ELISA). After protein stimulation of the cells, the IFN- γ secretion detection assay was performed according to the method described by the manufacturer (Miltinyi Biotech GmbH, Germany).

In brief, the protein-stimulated spleen cells were reacted with an anti-mouse IFN- γ mAb conjugated to leukocyte cell surface specific monoclonal Ab to allow binding of secreted IFN- γ to the cells. The secreted IFN- γ attached to the cells was then reacted with a second anti-IFN- γ mAb with a labeling agent appropriate to flow cytometry detection. Subsequently, the IFN- γ conjugated cells were stained either with anti-CD4 mAb-FITC conjugate or anti-CD8 mAb-FITC conjugate. Just prior to the flow cytometry analysis, propidium iodide was added to the cell suspension in order to exclude the dead cells from analysis. For the background control, non-immunized mouse spleen cells were stimulated with the proteins and processed similarly, while for the negative controls, immunized mouse spleen cells without protein stimulation were processed to determine non-specific IFN- γ secretion. For the positive controls, immunized mouse spleen cells were stimulated with 0.75 μg Concanavalin A (Con A) per 10^6 cells instead of the proteins. The percentage IFN- γ secreting T cells = $(\text{Number of IFN-}\gamma \text{ secreting T cells} / \text{Total number of T cells}) \times 100$.

Interleukin-2 (IL-2) Secretion Assay

Immunized mouse spleen cells were processed to determine secreted specific IL-2 according to the method of kit manufacturer (Mouse IL-2 Secretion

Assay, Miltenyi Biotech GmbH, Germany). The procedure was similar to that for the IFN- γ secretion determination; except anti-IL-2 mAb conjugates were used to trap secreted IL-2 for flow cytometry.

RESULTS

Construction of rBCG/HIV-1 gagE, rBCG/Codon Optimized gagE and rDIs/HIV-1 gagE, and Determination of the Amount of gag Protein Expressed

The BCG cell lysates of rBCG/HIV-1gagE and rBCG/codon optimized gagE, and the lysate of CEF infected with rDIs/HIV-1gagE were run electrophoretically and the proteins transferred onto nitrocellulose membrane were reacted with the anti-HIV-1 p24 mAb (Figure 1). The rBCG/HIV-1gagE expressed proteins which were reactive with the anti-gag mAb, resulting in a major protein band of approximately 55,000 Da, which was estimated to be the size of the whole gag protein. With regard to rDIs/HIV-1gagE, the same

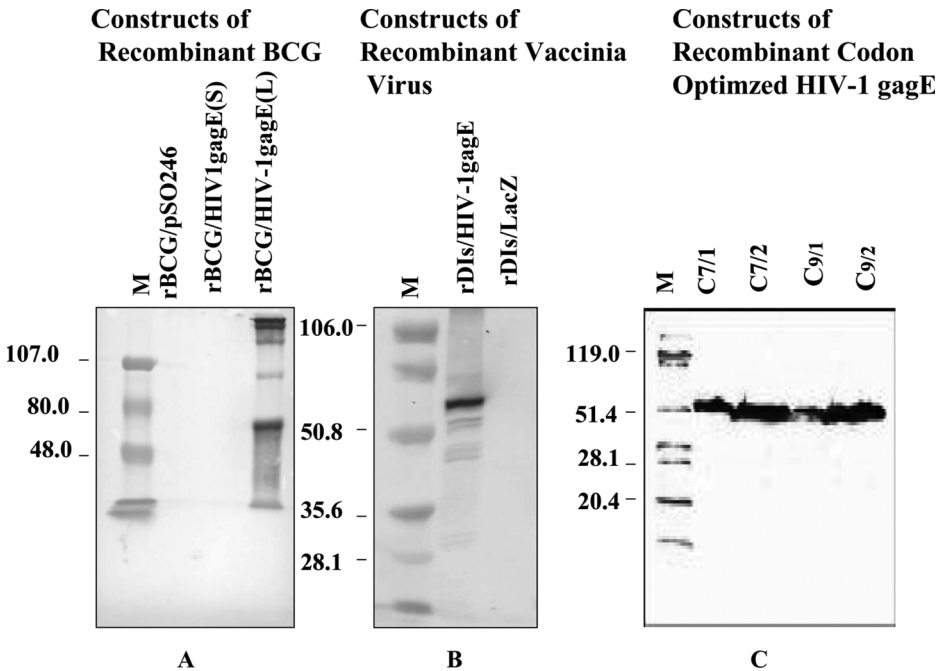


Figure 1: Western-blot analyses of the cell lysate (L) obtained from rBCG/HIV-1gagE in comparison with recombinant BCG transformed only with the DNA vector (rBCG/pSO246), and the culture supernatant (S) of rBCG/HIV-1gagE (A), the cell lysates obtained from rDIs/HIV-1gagE and recombinant Vaccinia virus transfected only with the DNA vector (rDIs/LacZ) infected cells (B), and various rBCG/codon optimized gagE clones (C7/1, C7/2, C9/1, and C9/2) (C), after reacting with the anti-HIV-1 p24 mAb (amino acids 287-304). M represents DNA molecular weight in kb.

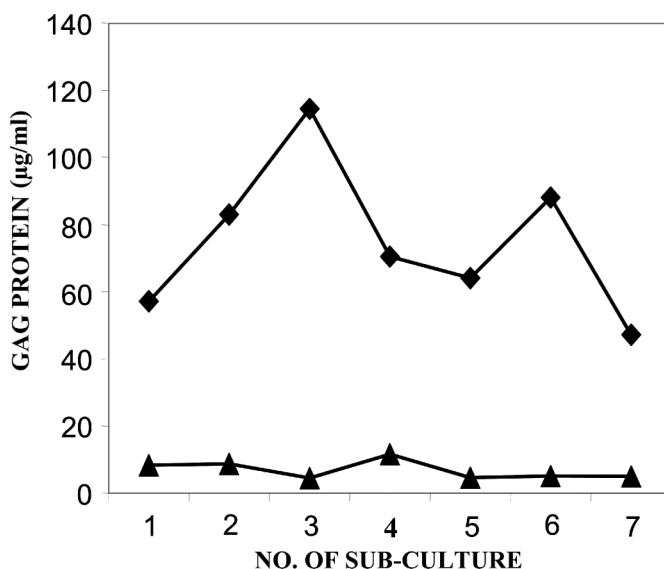


Figure 2: Amount of the gag protein expressions from seven different sub-cultures of rBCG/codon optimized gagE (■), and rBCG/HIV-1 gagE (▲).

HIV gene was inserted in-frame with the initiation codon of the p7.5 promoter of the plasmid, thus the HIV-1 gag protein was also expected to be expressed. The 55 KDa protein band was observed on the lane representing rDIs/HIV-gagE, while this was not detected in lysates from cells infected with rDIs/LacZ. For rBCG/codon optimized gagE, all clones handled in this test also showed immuno-reactive protein bands with the antibody. Additionally, after sub-culturing the codon optimized clones, expressed Gag was quantitated with the standard HIV-1p24 curve generated via the p24 ELISA. As shown in Figure 2, the gag protein produced from 7 different continuous sub-cultures of rBCG/codon optimized gagE ranged from 47.2–114.5 µg/ml while the wild-type constructs expressed 4.5–11.6 µg/ml culture; the averages of the codon usage constructs were ~11 times higher by similar BCG cell numbers which were maintained around $4.2\text{--}4.8 \times 10^{10}$ cells/litre.

CTL Responses in Mice Immunized with rBCG/Codon Optimized gagE Alone and the Prime-Boost of rBCG/Codon Optimized gagE with rDIs/HIV-1 gagE

To evaluate the CTL response induced by each immunization schedule, the percentage specific cell lysis obtained from the groups of negative control mice were used to subtract initially from those generated by the test groups with respective peptide stimulation. In general from the findings, all specific cell lyses of the negative control groups were found 0.0–3.5%. Since a single

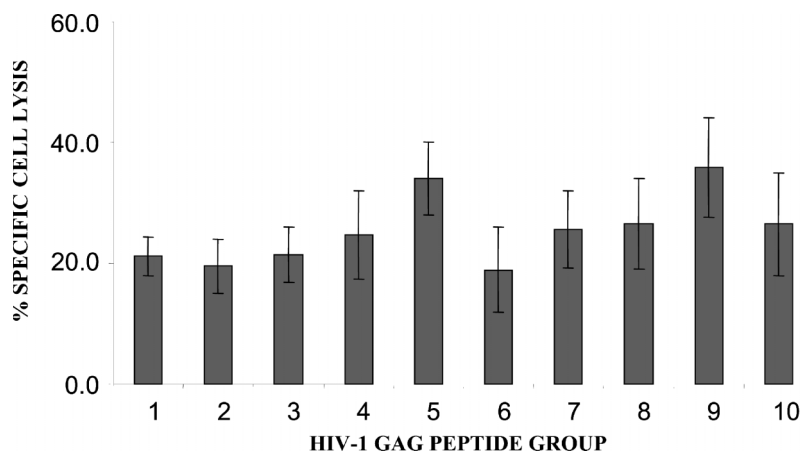


Figure 3: CTL induction as % specific cell lysis in mice immunized with a single injection of rBCG/codon optimized gagE against 10 different gag peptide groups as described in materials and methods. Error bars represent \pm standard deviation.

injection of the native rBCG/HIV-1gagE alone was found to induce gag-specific CTLs in moderate numbers in mice until 2 months after immunization, in our previous study (Promkhatkaew et al., 2009b), a similar experiment was investigated on the codon optimized construct in this study. As shown in Figure 3, with a single injection of 0.1 mg rBCG/codon optimized gagE s.c. for 70 days,, CTL were induced against all peptide regions along the gag protein, ranging from $18.9 \pm 7.0\%$ and $35.8 \pm 8.3\%$ specific cell lysis. These responses were quite similar to those induced by the single rBCG/HIV-1gagE injection and persistent within the same short period of immunization (data not shown).

The prime-boost with wild-type rBCG/HIV-1gagE as a priming Ag was compared versus rBCG/codon optimized gagE at the same dose, both boosted by rDIs/HIV-1gagE. As seen in Figure 4, both prime-boost regimens elicited CTL activity against all gag peptide groups. Higher activities ($20 \pm 3.1\%$ and $68 \pm 7.1\%$) were induced by the prime-boost having rBCG/HIV-1gagE, while lower activities ($13.1\% \pm 3.1\%$ and $45.5 \pm 7.8\%$) were observed from that of the codon optimized construct. Specific CTL induced by prime-boost immunizations either by the wild-type or codon-optimized BCG were drastically greater in some antigenic recognitions as those stimulated with the peptide groups 1, 2, 4, 6 and 8, than those induced by the single rBCG/codon optimized gagE or rBCG/HIV-1gagE injection alone (data not shown).

Cytokine Secretions

Various immunization schedules either with rBCG/HIV-1gagE or rBCG/codon optimized gagE were administered to mice, and splenic T cells secreting IFN- γ and IL-2 were measured. During cell counting by flow cytometry, dead cells were excluded by addition of propidium iodide, and T cells secreting IFN- γ

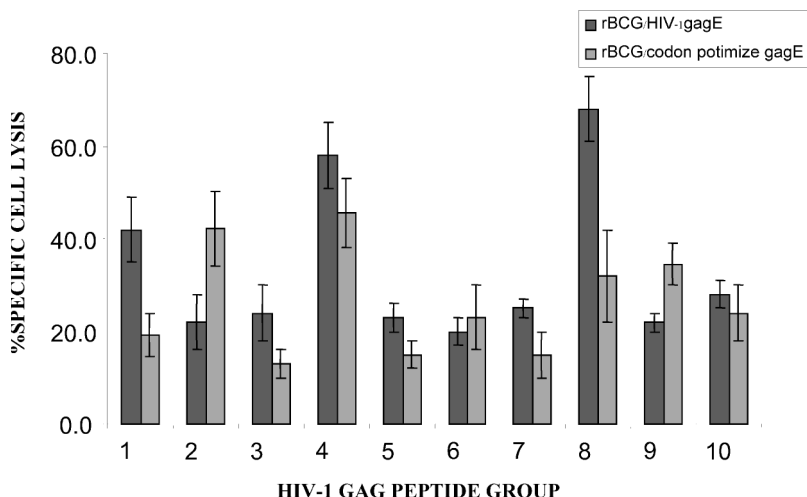


Figure 4: CTL induction as % specific cell lysis in mice immunized with the prime-boost immunizations having either rBCG/HIV-1gagE (dark bars) or rBCG/codon optimized gagE (grey bars) prime with rDIs/HIV-1gagE boost against 10 different gag peptide groups as described in materials and methods. Error bars represent \pm standard deviation.

or IL-2 were classified as CD4 or CD8 antigenic types by staining with the anti-CD4 and anti-CD8 antibodies.

Due to IFN- γ secretion, the positive (+) control is the maximum amount of CD4+ or CD8+ T cells from immunized spleen cells that could secrete IFN- γ when the cells were treated with Con A, while the negative (-) control means numbers of CD4+ or CD8+ T cells from the same immunized mice without any antigen stimulation. Non-immunized splenocytes were also tested by stimulation with respective proteins to provide the background CD4+ and CD8+ T cell numbers secreting non-specifically IFN- γ , and these were around 0.0%. A lymphocyte gate based on forward and side scatter (FSC/SSC) properties was activated prior to further gating to exclude dead cells.

Among various immunization regimens tested, the highest numbers of CD4+ and CD8+ T cells secreting IFN- γ were achieved from mice immunized with the prime-boost of 0.1 mg rBCG/codon optimized gagE and rDIs/HIV-1gagE as shown in Figures 5 and 6. In these studies, the splenocytes were separately stimulated with the mixtures of HIV-1 p17 and p24 proteins, and recombinant gag protein homologous to the vaccine constructs. The (+) controls, revealed the maximum numbers of CD4+ and CD8+ T cells capable of secreting IFN- γ as shown in Figure 5 as 0.46 and 0.24%, respectively; this prime-boost induced nearly as many CD4+ and CD8+ T cell IFN- γ , upon stimulations by both the p17 and p27 mixture and the gag protein as the (+) controls. All results generated by this prime-boost and others reflected from different immunization schedules including less rBCG/codon optimized gagE i.e. 0.05 and 0.02 mg were summarized as means \pm standard deviation as

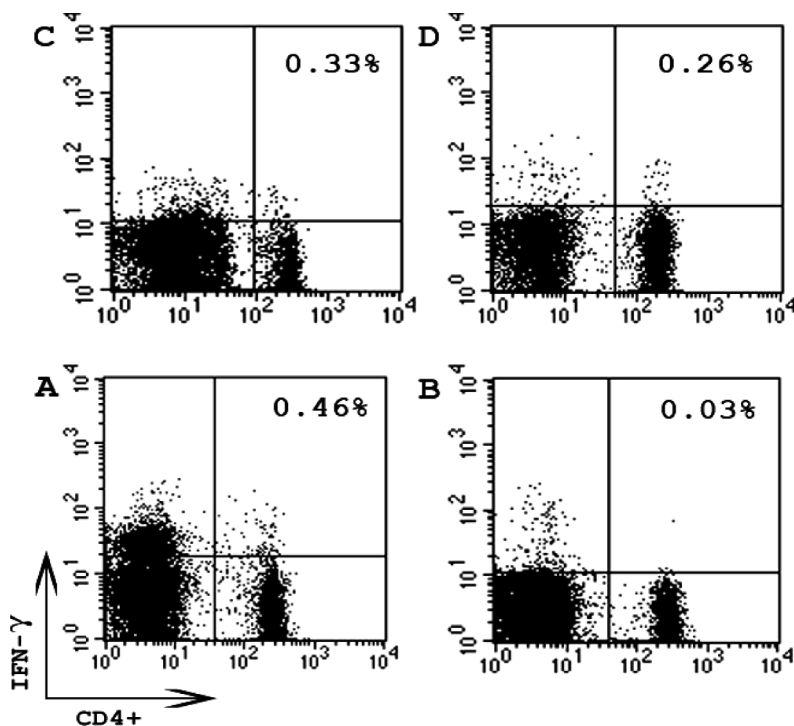


Figure 5: Scheme of flow cytometric analysis of IFN- γ secreting CD4⁺ T cells from mice immunized with 0.1 mg rBCG/codon optimized gagE prime with 10^6 pfu rDIs/HIV-1 gagE boost after stimulation during assay with the p17 + p24 protein mixture (C), and the recombinant gag protein (D). A and B are positive and negative controls as described in Materials and methods.

shown in Table 2. On contrary, the prime-boost with 0.1 mg wild-type rBCG/HIV-1gagE, or a single immunization of 0.1 mg rBCG/codon optimized gagE alone showed no IFN- γ secretion from either CD4⁺ or CD8⁺ T cells, as compared to (-) controls. When the amount of rBCG/codon optimized gagE was decreased to 0.05 mg, less IFN- γ secretion from both T cells was observed, and there was no detectable secretion when the dose was 0.02 mg in the prime-boost immunizations.

More IL-2 was also secreted from both CD4⁺ and CD8⁺ T cells after the prime-boost injection with 0.1 mg rBCG/codon optimized gagE stimulated by the p17 and p24 protein mixture and the recombinant gag protein as shown in Figures 7 and 8; this was not found in the groups immunized with 0.02 mg priming rBCG/codon optimized gagE or only 0.1 mg rBCG/codon optimized gagE alone, as the means with standard deviation are summarized in Table 3.

DISCUSSION

In the present study, the application of codon usage was applied to construct a recombinant BCG to express the same HIV-1 CRF01_AE gag as a vaccine

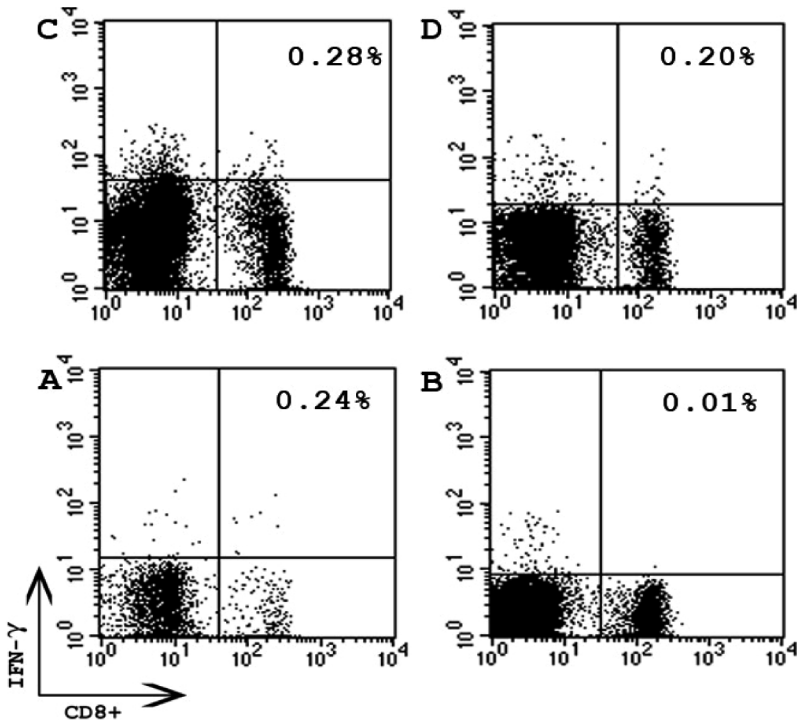


Figure 6: Scheme of flow cytometric analysis of IFN- γ secreting CD8+ T cells from mice immunized with 0.1 mg rBCG/codon optimized gagE prime with 10^6 pfu rDIs/HIV-1 gagE boost after stimulation during assay with the p17+p24 protein mixture (C), and the recombinant gag protein (D). A and B are positive and negative controls as described in materials and methods.

Table 2: CD4+ and CD8+ T cells secreting IFN- γ as a percentage of total CD4+ and CD8+ T cells after stimulation of mouse spleen cells obtained from various immunization regimens with the mixture of p17 and p24 proteins or the recombinant gag protein.

Immunization regimen	IFN- γ secreting T cells (%)			
	CD4+		CD8+	
	p17+p24	gag	p17 + p24	gag
0.1 mg rBCG/HIV-1 gagE + rDIs/HIV-1 gagE	0.05 \pm 0.01	0.03 \pm 0.01	0.03 \pm 0.01	0.06 \pm 0.01
0.1 mg rBCG/codon optimized gagE	0.02 \pm 0.01	0.01 \pm 0.01	0.03 \pm 0.01	0.03 \pm 0.00
0.1 mg rBCG/codon optimized gagE + 10^6 pfu rDIs/HIV-1 gagE	0.32 \pm 0.01	0.25 \pm 0.01	0.27 \pm 0.01	0.19 \pm 0.01
0.05 mg rBCG/codon optimized gagE + 10^6 pfu rDIs/HIV-1 gagE	0.21 \pm 0.01	0.13 \pm 0.01	0.15 \pm 0.01	0.07 \pm 0.01
0.02 mg rBCG/codon optimized gagE + 10^6 pfu rDIs/HIV-1 gagE	0.00 \pm 0.00	0.05 \pm 0.01	0.06 \pm 0.01	0.05 \pm 0.00
(+) Control	0.43 \pm 0.03		0.25 \pm 0.01	
(-) Control	0.03 \pm 0.00		0.02 \pm 0.01	

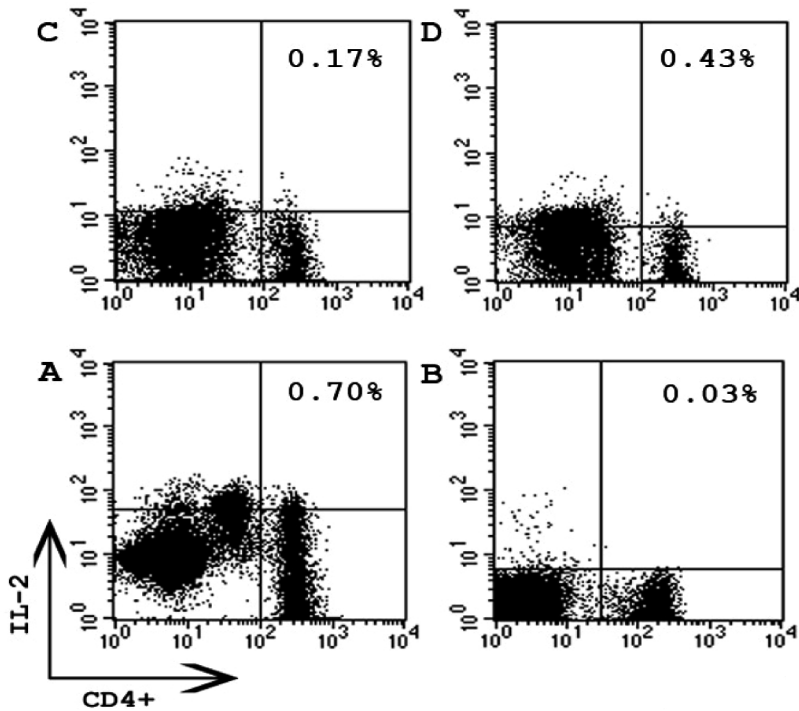


Figure 7: Scheme of flow cytometric analysis of IL-2 secreting CD4⁺ T cells from mice immunized with 0.1 mg rBCG/codon optimized gagE prime with 10^6 pfu rDIs/HIV-1 gagE boost after stimulation during assay with the p17+p24 protein mixture (C), and the recombinant gag protein (D). A and B are positive and negative controls as described in materials and methods.

candidate. To design a new optimized sequence conformed to the codons naturally appearing in the *Mycobacterium* spp., the codons of that of GenBank Acc. No. AY863146 were modified; 363 among the overall 493 codons or 73.6% were altered, most of them to contain C and G nucleotides compliant to the host cell as suggested in the website: *kazusa.or.jp*. The codon optimized construct produced more the gag protein (11 fold higher) than the wild-type recombinant BCG construct. Similarly to human genes, when A or T are replaced by G or C, higher protein expressions occur. In addition to this study, others have reported 10 fold increased protein production obtained from codon optimized HIV-1 gag-pol DNA vaccine (Kostsopoulou et al., 2000), even 4- to 966-fold higher found in various codon-modified gag plasmids (Megede et al., 2000).

With regard to CD8+CTL responses, the codon optimized BCG construct, whether as a single or a priming immunogen in the prime-boost vaccination with the recombinant Vaccinia virus containing HIV-1 gag, induced CTL activities in mice that recognized some gag eptiopes represented by respective peptide mixtures. Mice receiving the prime-boost showed much higher responses to that of single immunization of codon optimized construct,

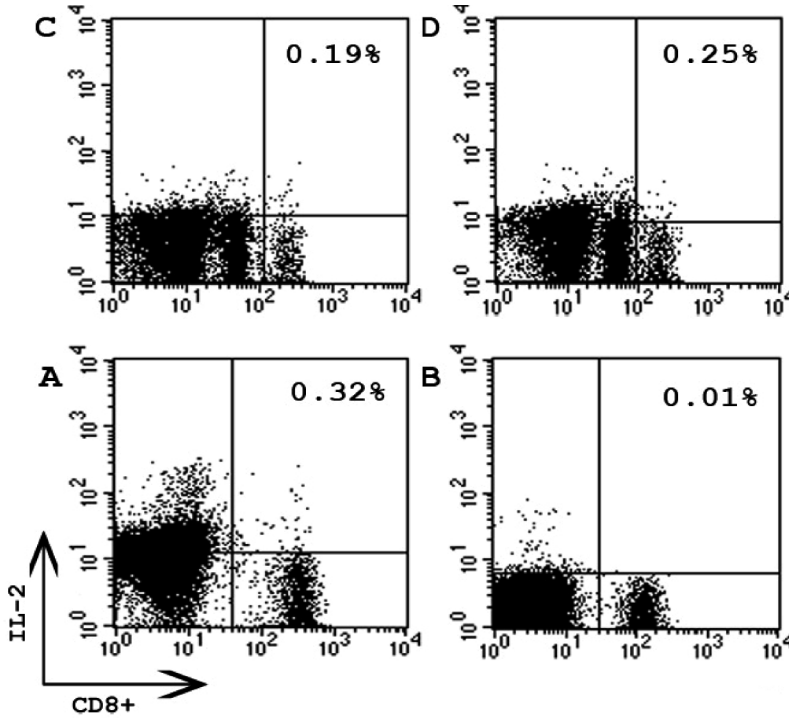


Figure 8: Scheme of flow cytometric analysis of IL-2 secreting CD8+ T cells from mice immunized with 0.1 mg rBCG/codon optimized gagE prime with 10^6 pfu rDIs/HIV-1 gagE boost after stimulation during assay with the p17 + p24 protein mixture (C), and the recombinant gag protein (D). A and B are positive and negative controls as described in materials and methods.

Table 3: Percentage CD4+ and CD8+ T cells secreting IL-2 over total CD4+ and CD8+ T cell numbers after stimulation of mouse spleen cells obtained from various immunization regimens with the mixture of p17 and p24 proteins or the recombinant gag protein.

Immunization regimen	IL-2 secreting T cells (%)			
	CD4+		CD8+	
	p17+p24	gag	p17+p24	gag
0.1 mg rBCG/codon optimized gagE	0.03 ± 0.01	0.09 ± 0.01	0.02 ± 0.01	0.01 ± 0.01
0.1 mg rBCG/codon optimized gagE + 10^6 pfu rDIs/HIV-1 gagE	0.18 ± 0.01	0.41 ± 0.02	0.18 ± 0.01	0.24 ± 0.01
0.02 mg rBCG/codon optimized gagE + 10^6 pfu rDIs/HIV-1 gagE	0.09 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.04 ± 0.01
(+) Control	0.69 ± 0.01		0.36 ± 0.04	
(-) Control	0.02 ± 0.01		0.02 ± 0.01	

particularly in some of antigenic recognitions by stimulation with peptide groups 1, 2, 4, 6 and 8. This indicates more strikingly success of our vaccine candidates for CTL induction either as BCG-based codon modified or native gag genes as the prime antigen with the boost of Vaccinia virus-based candidate, supporting our previous studies.

Since it has been shown that in BCG vaccinated human subjects, reactive antigen-specific CD8+ T-cell immunity was detected secreting IFN- γ in addition to potent CTL activity (Smith et al., 1999), and specific IFN- γ has ever been found responsive by Th-1 CD4+ T cells in mice infected with live BCG (Huygen et al., 1994), we also focused on cytokine induction whether by CD4+ or CD8+ T cells. It is obvious that in the prime-boost regimen a dose of 0.1 mg rBCG/codon optimized gagE was required, regardless of whether IFN- γ and IL-2 secretions via CD4+ and CD8+ T cell recognition of whether the subtype B p17 and p24 mixture or a homologous recombinant CRF01_AE gag protein was used to stimulate.

The IFN- γ secretions were in a less when the codon optimized immunogen was reduced to 0.05 mg, and no secretion with 0.02 mg. The priming wild-type rBCG/HIV-1gagE at a dose of 0.1 mg did not induce the same quantity of cytokine secretion. Similar findings were also observed in IL-2 secretion, significantly higher IL-2 releases were induced by 0.1 mg than by 0.02 mg rBCG/codon optimized gagE, but not in construct injected alone. These may prove the effect of robust higher antigen expression of the codon optimized candidate than the wild-type HIV-1 acquired during immunization. A single injection of codon optimized construct in the same amount (0.1 mg) had no effect on IFN- γ or IL-2 secretion from CD4+ and CD8+ T cells. This suggests subsequent immunization with another antigen is required to boost up or enhance existing primed T cells to proliferate or be reactivated. But the initial immunization might need more antigen presentation, hence the codon modified immunogen is superior.

It was believed that a better subsequent antigen might be a live virus. To support the viral influence, there has been a report describing cells infected with recombinant virus encoding HIV-1 genes activated and expanded both CD4+ T cells and CTL (Engelmayer et al., 2001). Similar to the present study, Im et al. (2007) have shown that the priming and boosting regimen with a recombinant lysine auxotroph of BCG and a modified vaccinia virus Ankara (MVA) expressing HIV-1 clade A has provided CD8+ and CD4+ T cell responses as well as protection against surrogate virus challenge, while we investigated further the recombinant BCG with modification of HIV codons in compliance with the host cell.

In this study, we found that CD8+ CTL and cytokine secretions as well as CD4+ T helper cell responses could be induced by the prime-boost comprising rBCG/codon optimized gagE and rDIs/HIV-1gagE, especially, cytokine secretions from both T cells were restricted to a particular priming dose. Since the

macrophage is a target cell for BCG, it may facilitate the activation of CD4+ Th1 cells through antigen presenting cells, and these activated Th1 cells may then induce the expression of Th1 cytokines, especially IFN- γ (Nathan et al., 1983). IL-2 is a growth factor for antigen-stimulated T cells and is responsible for T cell clonal expansion after antigen recognition. Theoretically, IL-2 is produced by CD4+ T cells, and to a lesser amount, by CD8+ T cells. Our live-vector based HIV vaccine candidates and immunization schedules may be beneficial in that the antigen-specific CD4+ T cells are required for secondary expansion of memory CD8+ T lymphocytes (Janssen et al., 2003). Moreover, both CD4+ Th1 and CD8+ T cell secreting cytokines including IFN- γ and IL-2, would assist in the full differentiation and maturation of CTL (Delves et al., 2000), as was the focus of strategic immune response of our vaccine candidates.

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ABBREVIATIONS

HIV human immunodeficiency virus
IFN- γ interferon gamma
IL-2 interleukin 2
CTL cytotoxic T lymphocyte

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