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Novel codon-optimized GM-CSF gene as an adjuvant to enhance the immunity of a DNA vaccine against HIV-1 Gag

Jian-Tai Qiu [a,b,c,*], Ting-Chang Chang [a], Cheng-Tao Lin [a], Yi-Ming Chen [c], Frank Q. Li [d], Yung-Kuei Soong [a], Chyong-Huey Lai [a,*]

[a] *Department of Obstetrics and Gynecology, Chang Gung Memorial Hospital, Taoyuan 333, Taiwan*
[b] *Department of Life Science, Chang Gung University College of Medicine, Taoyuan 333, Taiwan*
[c] *AIDS Prevention and Research Center, National Yang-Ming University, Taipei, Taiwan*
[d] *Vaxim Inc., Rockville, MD 20850, USA*

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Abstract

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a potent immunomodulatory cytokine. Here we generated a novel codon-optimized murine GM-CSF gene as an adjuvant. The codon-optimized GM-CSF gene significantly increased protein expression levels in all cells tested. Although injection of the wild-type GM-CSF plasmids adjuvanted HIV-1 Gag DNA vaccine induced detectable immune responses, co-administration of plasmids encoding the codon-optimized GM-CSF sequence with the DNA vaccine resulted in a strong antibody and CTL responses and a protective immune response against infection with recombinant vaccinia virus expressing HIV-1 Gag. This novel codon-optimized GM-CSF gene offers a practical molecular strategy for potentiating immune responses to vaccines as well as other immunotherapeutic strategies.

Keywords: GM-CSF; DNA vaccine; HIV-1; T-lymphocytes; Immunity

1. Introduction

DNA vaccines have emerged as an attractive approach for generating antigen-specific immunotherapy (for reviews, see [1,2]). Such vaccines have been highly effective in mice; however, their efficiency is restricted in larger animals. Therefore, multiple strategies have been developed for strengthening DNA vaccine potency. Much of the focus has been on identification of the most effective delivery system [3–5], codon optimization of viral antigens [6,7], various prime-boost protocol [5,8], coadministration with CpG oligonucleotides [9], and co-injecting cytokines [10–12].

Among the various cytokines tested, granulocyte-macrophage colony-stimulating factor (GM-CSF) is one of the most potent, and its use has achieved some success in tumor models [13], malaria [14] and HIV [15–17]. The key to the role of GM-CSF as an immunomodulatory is its ability to recruit and activate functional antigen-presenting cells (APC) [18,19] such as dendritic cells, the most potent activators of T cells [20].

Because the GM-CSF gene expression is highly regulated at multiple levels [21–23], the protein expression of native GM-CSF gene is poor and in a tissue-specific as well as an activation-dependent manner [21–23]. Genes that are expressed with high frequency include a bias toward particular codons. The codons associated with highly expressed genes also vary with species [24,25]. These differences, such as A and T preferences in the third position of a codon observed in bacteria, have been incorporated into human genes for enhancing expression of heterologous genes in

* Corresponding authors at: Department of Obstetrics and Gynecology, Chang Gung Memorial Hospital, 5 Fu-Shin St., Kueishan, Taoyuan 333, Taiwan. Tel.: +886 3 328 1200; fax: +886 3 328 4320.
E-mail addresses: jtqiu@cgmh.org.tw (J.-T. Qiu), laich46@cgmh.org.tw (C.-H. Lai).

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microorganisms [26,27]. A number of studies have found that there is a good correlation between the codon bias of a gene and its level of expression [28–30].

GM-CSF is species specific and human GM-CSF has no biological effects on mouse cells. We optimized condon of murine GM-CSF to evaluate the HIV-1 Gag-specific immune response in a mouse model system. In this current study, we used total gene synthesis to develop an optimized murine GM-CSF construct that expresses the proteins 60-fold higher than the native construct in a murine cell line. Using a DNA vaccination model, we determined that immunization with optimized GM-CSF in combination with HIV-1Gag DNA constructs resulted in a significant enhancement of Ag-specific primary antibody responses and IFN-γ secretion, and strong induction of $CD8^+$ T cell responses. We also repeated and confirmed that low dose and shorter DNA vaccination followed by recombinant vaccinia virus-Gag challenge allowed us to establish a novel physiological method for measuring the memory $CD8^+$ cytotoxic activity in DNA-vaccinated mice without in vitro restimulation [31]. Additionally, in this report, we investigate the ability of the codon-optimized GM-CSF construct to augment the secondary antibody responses and $CD8^+$ cytotoxic activity elicited by an HIV-1 Gag DNA vaccine in mice. This current study demonstrated the optimized gene sequences encoding murine GM-CSF as a candidate adjuvant for vaccine or immunotherapeutic studies.

2. Materials and methods

2.1. Plasmids

The HIV-1 Gag expression vector (pGAG) used in these experiments was pGAGINS, which has been previously described [43,44]. The wild-type GM-CSF expression vector used in these experiments was amplified from the plasmid pCEP4/GM-CSF generously provided by Dr. Mi-Hua Tao [49] (Academia Sinica, Taipei, Taiwan), by PCR using the sense primer (5′-CCCCTCGCGAGCGCACCCACCCG-CTCACCC-3′, with the site *Nru*I underlined) and the antisense primer (5′-CCGAATTCGTTAACCTTTTTGGACTG-GTTTTTTGCATTC-3′, with the *Eco*RI site underlined). The PCR product was digested with *Nru*I and *Eco*RI and cloned into the vector pcDNA3.1- (Invitrogen, Calsbad, CA) downstream from the human CMV immediate-early promoter sequence, using *Pme*I and *Eco*RI sites to generate pwtGM-CSF. To generate the codon-optimized form of murine GM-CSF, pcGM-CSF (GenBank accession no. AY950559), we changed the codon usage to that utilized by highly expressed human genes as described previously [6,32]. The gene was created by total gene synthesis (Aptagen Inc., Herndon, VA) and was inserted into the *Xba*I and *Xho*I sites of pcDNA3.1- (Invitrogen) downstream from the promoter sequence. The correct plasmids were identified by DNA sequencing.

2.2. Cell lines and transfections

NIH 3T3, COS-7, 293 and HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U of penicillin per ml, and 100 mg of streptomycin per ml and were passaged upon confluence. p815 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum–100 U of penicillin/ml–100 mg of streptomycin/ml. HeLa cells are human cervical epithelial carcinoma cells. NIH 3T3 cells are mouse fibroblasts. CT-26 cells are murine colon carcinoma cells with an *H-2d* MHC background. COS-7 cells were transiently transfected by the DEAE-dextran method as previously described [44]. NIH3T3, CT-26, and HeLa cells were transfected by the lipofectin method as suggested by the manufacturer (Life Technologies, Gaithersburg, MD). At 24 h after transfection, the transfected CT-26 cells were washed once with complete medium and selected with the antibiotic G418 (1.2 mg/ml) for 2–3 weeks. The established CT-26 cell lines were maintained in complete medium and 0.4 mg of G418/ml. Cell culture reagents were obtained from Life Technologies (Gaithersburg, MD). NFS-60 is a murine myelogenous leukemia cell line derived from an NFS/N mouse and often used in GM-CSF bioassay [50]. The cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum in the presence of 1 U/ml recombinant mouse GM-CSF (R&D, Minneapolis, MN).

2.3. Immunoblotting

Three days after transfection, cell lysates were prepared as previously described [44]. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out using standard methods. GAPDH was the marker of choice as loading control in Western blots and protein normalization. Proteins were transferred to nitrocellulose filters (Schleicher and Schuell). The blots were stained by using rabbit anti-murine GM-CSF (PeproTech, Rocky Hill, NJ) in a PBS solution with 1% nonfat dried milk. The secondary antibody was an alkaline phosphatase-conjugated Goat anti-rabbit antibody (KPL, Rockville, MD), and staining was carried out with a solution containing 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitroblue tetrazolium prepared from chemicals obtained from Sigma (St. Louis, MO).

2.4. Measurement of GM-CSF secretion

The levels of GM-CSF secreted from the cells were measured by a biological assay or an enzyme-linked immunosorbent assay (ELISA). The NFS-60 cells that proliferated in response to GM-CSF were used for a biological assay [50], and cell proliferation was measured by a $[^3H]$-thymidine incorporation assay in triplicate wells. A commercially available ELISA kit with a sensitivity of 5 pg GM-CSF/ml (Endogen, Woburn, MA) was used to measure the GM-CSF levels. Culture supernatant diluted 100-, 200- and 400-fold was used

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in the assays. The data were further interpolated in a standard curve obtained from recombinant GM-CSF (R&D) of known concentrations, and the levels of GM-CSF were determined.

2.5. Vaccination of mice

Plasmids were propagated in bacterial strain JM109 and were purified with a QIAGEN (Chatsworth, CA) Endo-free Maxiprep Kit. The plasmids were resuspended at 1 mg/ml in sterile normal saline solution and were stored at −20 °C until the day of immunization. The Gag and GM-CSF plasmids were equally mixed together to reach a total of 100 μg per each immunization. Female BALB/c mice, 6–8 weeks old (purchased from Harlan, Frederick, MA), were divided into four groups of five mice each, and the mice were each immunized with a total of 100 μg of DNA by i.m. injection with 50 μl of plasmid DNA in separate sites in both side quadriceps, followed by two i.m. booster vaccinations at 2-week intervals as previously described [31].

2.6. Assay for anti-Gag antibodies

BALB/c mice (five per group) were intramuscularly injected three times with 100 μg of plasmid DNA each at 0, 2, and 4 weeks. Anti-Gag antibodies were measured at weeks 2, 4 and 6. Serum was collected from each group of mice injected with the various DNA constructs. It was then pooled and analyzed by immunoblotting using purified immature viral particles containing unprocessed Gag p55 or mature HIV-1 virions from H9 cells [44]. Quantitation of immunoblots was carried out with the NIH Image Program, version 1.52. The immunoblots were scanned using an Eagle Eye II (Stratagene, Garden Grove, CA), and digital image analysis was carried out with NIH Image. The intensity of the Gag-specific antibody response for pGAG + cGM-immunized mice at week 2 was designated as 100%.

2.7. Cytokine ELISPOT assay

Ninety-six-well filtration plates (Millipore, Bedford, MA) were coated overnight at 4 °C with 50 μl (10 μg/ml) of anti-mouse IFN-γ (R46A2; BD Pharmingen, San Diego, CA) in sterile PBS. The plates were blocked for 2 h at 37 °C with sterile RPMI 1640 containing 10% fetal calf serum and 1% bovine serum albumin and were washed three times with sterile PBS. Various dilutions of splenocytes in 200 μl of complete medium with or without MHC class I-restricted p24 peptide (aa AMQMLKETI) were placed in each well and incubated at 37 °C for 24 h. Plates were washed with PBS containing 0.025% Tween-20 and were overlaid with 50 μl (5 μg/ml) of biotinylated anti-mouse IFN-γ (XMG1.2; BD Pharmingen). The plates were washed six times with PBS containing 0.025% Tween-20 and were treated with 1.25 μg of avidin-conjugated alkaline phosphatase (Sigma, St. Louis, MO) per ml for 2 h at room temperature. After a final wash with PBS, IFN-γ spot-forming cells were detected by the addition of BCIP-nitroblue tetrazolium solution (Sigma) and were counted with a stereomicroscope.

2.8. CTL assay

Female BALB/c mice (10 per group), 6–8 weeks old, were immunized by i.m. injection with 100 μg of plasmid DNA each at 0, 2, and 4 weeks. The mice were sacrificed at week 6. The spleens were removed from the immunized mice, compressed through sterile nylon mesh with a rubber stopper, and then washed twice with RPMI 1640. Splenic mononuclear cells were isolated by centrifugation through Ficoll-Hypaque (Pharmacia, North Peapack, NJ) discontinuous density gradients. The harvested cells were centrifuged for 10 min in a Sorvall H-1000B rotor at 1000 rpm (200 × *g*) and resuspended in complete culture medium (RPMI 1640 medium supplemented with 10% fetal calf serum, 50 mM 2-mercaptoethanol, 2 mM L-glutamine, 100 U of penicillin per ml, and 100 mg of streptomycin per ml) for the in vitro restimulation. Cell viability was determined by trypan blue exclusion. The stimulator cells (5×10^6 ml^{-1}), harvested from naive mice, were pulsed with 10 mg of MHC class I-restricted p24 peptide (p7g, aa 199 AMQMLKETI 207) per ml for 1 h at 37 °C and then irradiated at 3000 rads. The cells were pelleted and washed three times with RPMI medium. The effector cells in 24-well plates were incubated with stimulator cells at an effector-to-stimulator (E/S) ratio of 5/1 for 6 days in culture medium containing 15 U of IL-2/ml. To measure the specific lysis of these target cells, we used the lactate dehydrogenase (LDH) release assay. This assay yields results similar to those obtained with the standard chromium release assays but does not require the use of radioisotopes. In 96-well round-bottom plates, target cells were incubated with effector cells at various effector-target ratios for 5 h in phenol red-free RPMI 1640 containing 3% fetal calf serum, 2 mM L-glutamine, 100 U of penicillin per ml, and 100 mg of streptomycin per ml. The target cells (p815, 10^7 cells/ml) were incubated with 10 μg of MHC class I-restricted p24 peptide (aa 199 AMQMLKETI 207) per ml for 1 h at 37 °C and then washed three times with assay medium. Fifty microliters of the supernatant per well was then transferred to 96-well plates, and lysis was determined by measuring LDH release by using the Cytotox 96 assay kit (Promega Corp., Madison, WI). The released LDH converts the added substrate tetrazolium salt into a red formazan product, where the amount of color is proportional to the number of lysed cells. The absorbance values from supernatants were recorded at 490 nm on an ELISA microplate reader. The percentage of specific lysis of the peptide-pulsed p815 target cells for a given effector cell sample was calculated by the following formula: specific lysis = (optical density [OD] of experimental LDH release − OD of effector cell spontaneous LDH release − OD of target cell spontaneous LDH release)/(maximum target cell LDH release − OD of target cell spontaneous LDH release) × 100%. All determinations were performed in triplicate.

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2.9. Intracellular cytokine staining of splenocytes from challenged mice

Female BALB/c mice (10 per group), 6–8 weeks old, were immunized by i.m. injection with 50 μg of plasmid DNA each at weeks 0 and 2. Thirty-five days after the last DNA immunization, mice were intraperitoneally (i.p.) inoculated with 10^7 PFU of recombinant vaccinia viruses containing the HIV-1 gag gene (vP1287, catalog no. 3542; NIH AIDS Research and Reference Reagent Program). Four days later, the splenocytes from naive or challenged mice were harvested and incubated with or without the p24 peptide (aa AMQMLKETI). The p24 peptide was added at a concentration of 2 μg/ml for 24 h, and Golgistop (Pharmingen) was added 6 h before the cells were harvested. The cells were then washed once in fluorescence-activated cell sorter buffer and stained with phycoerythrin-conjugated monoclonal rat anti-mouse $CD8^+$ antibody (Pharmingen). Intracellular cytokine staining was carried out by using the Cytofix/Cytoperm kit as suggested by the manufacturer (Pharmingen). Fluorescein isothiocyanate-conjugated anti-IFN-γ antibodies and the immunoglobulin isotype control antibody (rat IgG1) were all purchased from Pharmingen. Analysis was done on a Becton Dickinson FACScan with CELLQuest Software (Becton Dickinson Immunocytometry Systems, Mountain View, CA).

2.10. Vaccinia virus titer in the ovaries of challenged mice

Thirty-five days after the final DNA vaccination, the mice (10 per group) were challenged i.p. with 10^7 PFU of vaccinia virus expressing HIV-1 Gag (vP1287). Four days after the challenge, the mice were sacrificed and their ovaries were removed, homogenized, sonicated, and assayed for vP1287 titer by plating serial 10-fold dilutions on a plate of BSC-1 indicator cells. After 2 days of culture, the medium was removed, the BSC-1 cell monolayer was stained with 0.1% crystal violet (Sigma) for 5 min, and the number of plaques per well was counted.

3. Results

3.1. Construction of murine GM-CSF expression vectors

The native sequence coding for the 153 amino acids (aa) of murine GM-CSF (GenBank accession no. X02333; Fig. 1, upper sequence) was amplified by PCR and cloned into pcDNA3.1(−) to generate pwtGM-CSF. To generate pcGM-CSF, the codon-optimized form of murine GM-CSF, we changed the codon usage to that utilized by highly expressed human genes as previously described [6,32]. The resulting optimized murine GM-CSF encode a protein without any amino acid change (Fig. 1, lower sequence; GenBank accession no. AY950559). The resulting 480-bp gene cassette included the *Xba*I and *Xho*I cloning sites and was constructed synthetically. This optimized murine GM-CSF sequence was cloned into the *Xba*I and *Xho*I restriction sites of the eukaryotic expression vector pcDNA3.1(−) that employs the cytomegalovirus (CMV) immediate-early enhancer/promoter and bGH terminator (Invitrogen, San Diego, CA), resulting in the plasmid pcGM-CSF. The CMV promoter provides a high level of constitutive expression in a range of mammalian cells. The BGHpA signal provides efficient transcription termination and polyadenylation of mRNA.

3.2. Comparison of murine GM-CSF expression in transfected cells

We initially tested the protein expression of wild-type and codon-optimized murine GM-CSF expression vectors transfected into mouse (NIH3T3), monkey (COS-7) and human (HeLa) cells. The cell lysates from transfected cells were analyzed by immunoblotting with a rabbit anti-murine GM-CSF antibody using GAPDH as a loading control in Western blots and protein normalization. GM-CSF protein was detected in transfected NIH3T3, COS-7 and HeLa cells with the pcGM-CSF constructs (Fig. 2A). Also, GM-CSF protein was hardly detected in pwtGM-CSF-transfected NIH3T3 cells (Fig. 2A, lane 1). The secreted GM-CSF protein was detected in culture supernatants of pcGM-CSF-transfected cells by the rabbit anti-murine GM-CSF antibody (Fig. 2B, bottom panel). As expected, no GM-CSF protein was detected in culture supernatants of pcDNA3.1-transfected cells. The pcGM-CSF-transfected cells produced a greater amount of secreted GM-CSF proteins (11,605 pg/ml in NIH3T3, 12,048 pg/ml in COS-7 and 13,250 pg/ml in HeLa cells) than the pwtGM-CSF-transfected cells (Fig. 2B; 158 pg/ml, 205 pg/ml and 210 pg/ml, respectively). These results suggest that the novel codon-optimized murine GM-CSF coding sequences increased not only GM-CSF expression but also increased very significantly the release of the protein in mouse (NIH3T3), monkey (COS-7) and human (HeLa) cells. The same evidence of increased expression of pcGM-CSF was also demonstrated in murine TC-1, CT-26, and human 293 cells (data not shown). We are testing this novel GM-CSF sequence in primary cells, including naïve T cells and dendritic cells.

3.3. Anti-Gag antibody responses in mice immunized with naked DNA vaccine

To evaluate the adjuvant effect of the novel GM-CSF gene in primary humoral immune responses, BALB/c mice were intramuscularly injected with a total of 100 μg of plasmid DNA three times, at 0, 2 and 4 weeks. Anti-Gag antibodies were measured at weeks 2, 4 and 6. Sera were collected from the mice injected with the different DNA constructs, pooled and analyzed by immunoblot using purified immature viral particles containing unprocessed Gag p55 or mature HIV-1 virions from H9 cells or by ELISA by using purified Gag p55

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DNA alignment Between Original (wtGM-CSF) and optimized sequence (cGM-CSF)

GenBank Access number: X02333 and AY950559

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                M  A  H  E  R  K  A  K  V  L  R  R  M  W  L  Q  N  L  L  F
  cGM:   1      atggctcacgagcggaaggctaaggtgctgcgcagaatgtggctgcagaacctgctgttc  60
wt-GM:   138    atggcccacgagagaaaggctaaggtcctgaggaggatgtggctgcagaatttacttttc  197

                L  G  I  V  V  Y  S  L  S  A  P  T  R  S  P  I  T  V  T  R
  cGM:   61     ctgggcatcgtggtgtacagcctgagcgcccccaccagaagccccatcaccgtgaccaga  120
wt-GM:   198    ctgggcattgtggtctacagcctctcagcacccacccgctcacccatcactgtcacccgg  257

                P  W  K  H  V  E  A  I  K  E  A  L  N  L  L  D  D  M  P  V
  cGM:   121    ccctggaagcacgtggaggccatcaaggaagctctgaacctgctggacgacatgcccgtg  180
wt-GM:   258    ccttggaagcatgtagaggccatcaaagaagccctgaacctcctggatgacatgcctgtc  317

                T  L  N  E  E  V  E  V  V  S  N  E  F  S  F  K  K  L  T  C
  cGM:   181    accctgaacgaggaggtggaggtggtgagcaacgagtttagctttaagaagctgacctgc  240
wt-GM:   318    acattgaatgaagaggtagaagtcgtctctaacgagttctccttcaagaagctaacatgt  377

                V  Q  T  R  L  K  I  F  E  Q  G  L  R  G  N  F  T  K  L  K
  cGM:   241    gtgcagacccggctgaagatcttcgagcagggactgcggggcaactttaccaagctgaag  300
wt-GM:   378    gtgcagacccgcctgaagatattcgagcagggtctacggggcaatttcaccaaactcaag  437

                G  A  L  N  M  T  A  S  Y  Y  Q  T  Y  C  P  P  T  P  E  T
  cGM:   301    ggagccctgaacatgaccgccagctactaccagacctactgccctcccacacccgagacc  360
wt-GM:   438    ggcgccttgaacatgacagccagctactaccagacatactgccccccaactccggaaacg  497

                D  C  E  T  Q  V  T  T  Y  A  D  F  I  D  S  L  K  T  F  L
  cGM:   361    gactgtgaaacccaggtgaccacctacgccgactttatcgacagcctgaagaccttcctg  420
wt-GM:   498    gactgtgaaacacaagttaccacctatgcggatttcatagacagccttaaaacctttctg  557

                T  D  I  P  F  E  C  K  K  P  V  Q  K  &  L  E  R
  cGM:   421    accgacatccccttcgagtgtaagaagcccgtgcagaagtgactcgagcgg
wt-GM:   558    actgatatccccttgaatgcaaaaaaccagtccaaaaatga
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Fig. 1. Sequence comparison between the wild-type murine *GM-CSF* sequence (wt-GM; GenBank accession no. X02333) and the codon-optimized GM-CSF sequence (c-GM; GenBank accession no. AY950559). The amino acid sequence is 100% homologous for each sequence.

recombinant protein expressed from baculovirus (data not shown). As shown in Fig. 3, the pcGM-CSF elicited a higher and more rapid antibody response than did the wild-type GM-CSF. Specific antibodies against HIV-1 p24 in Balb/c mice immunized with pcGM + Gag vectors were detectable 2 weeks after a single vaccination. In addition, no antibody response was detectable at 2 weeks in the mice immunized with pwt-GM + Gag DNA (Fig. 3). At 4 and 6 weeks, antibody levels in the pcGM + Gag -immunized group were still 4- to 6-fold higher than those in mice immunized with pwt-GM + Gag. No anti-Gag antibodies were detected in mice immunized with pcDNA3.1. Administration of pGAG plasmids alone did not induce detectable IgG antibody responses at weeks 2 and 4(data not shown). The co-administration of the wild-type GM-CSF plasmids induced only a modest increase in total IgG anti-Gag serum titers. By comparison, co-administration of the codon-optimized GM-CSF plasmids significantly increased total IgG serum titers.

3.4. ELISPOT assay in DNA-vaccinated mice

To test the primary $CD8^+$ cellular immune responses at single-cell level, mice were immunized with the various DNA constructs as described above. At week 6, splenocytes from the mice in each group were harvested and pooled. The IFN-γ ELISPOT assay specific to HIV-1 Gag peptide was measured following 24-h antigen stimulation. As expected, those plasmids that did not express Gag p55 in vitro, such as pcDNA3.1, did not elicit an antigen-specific $CD8^+$ T cell response against HIV-1 Gag (Fig. 4). A 3-fold increase in the number of cells

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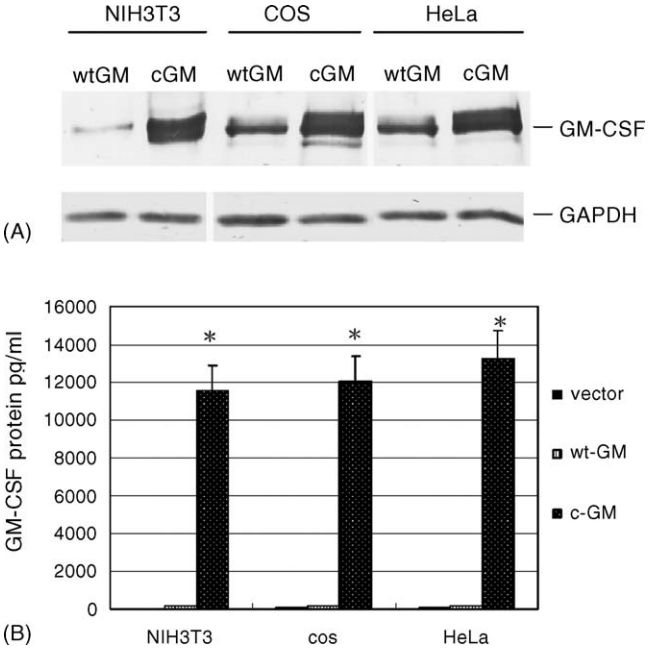

Fig. 2. Wild-type (wt-GM) and codon-optimized (c-GM) murine GM-CSF expression in transfected cells. The NIH3T3, COS-7 and HeLa cells were transfected with control vector pcDNA3.1-, wild-type and codon-optimized murine GM-CSF DNA constructs. Cell lysates from transfected cells were separated by SDS-PAGE, transferred to nitrocellulose membranes, and analyzed by immunoblotting with a rabbit anti-murine GM-CSF antibody (A, top panel). GAPDH is the marker of choice as loading control in Western blots and protein normalization (data not shown). Culture supernatants were collected three days after transfection. Soluble GM-CSF released from transfected cells was monitored by ELISA (B, bottom panel). The codon-optimized GM-CSF was 60-fold increased in expression according to the ELISA. $^{*}P < 0.001$ by paired Student *t*-test. These data are representative of three independent experiments.

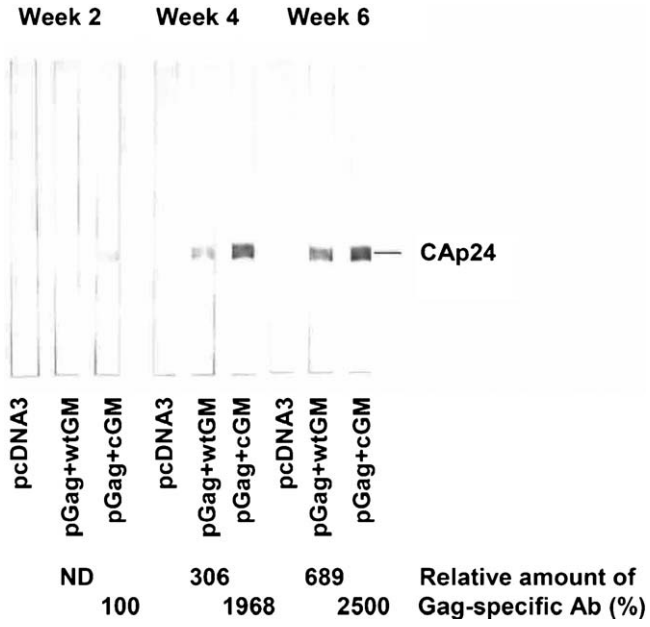

Fig. 3. Anti-HIV-1 Gag antibody responses. Balb/c mice (five per group) were intramuscularly injected three times with total 100 μg of mixture of the plasmid DNA constructs at weeks 0, 2, and 4. Anti-HIV-1 Gag antibody responses were measured at weeks 2, 4 and 6. Sera were collected from five mice in each group and analyzed by immunoblotting with purified mature HIV-1 virions. Relative amount of Gag-specific IgG antibody was measured by the NIH Image Program. The intensity of the Gag-specific antibody response for pGAG + cGM immunized mice at week 2 was designated as 100%. These data are representative of four independent experiments.

secreting Ag-specific IFN-γ to p7g peptide was detected in mice immunized with the addition of wild-type GM-CSF plasmids (500 SFU versus 170 SFU, Fig. 4, lane 3 versus lane 2). The group of vaccinated mice that received pcGM + Gag developed a slightly higher level of HIV-1 Gag-specific $CD8^{+}$ activity than those that received pwt-GM + Gag. These results showed that intramuscular injection of the mixture of pGAG and codon-optimized GM-CSF plasmids produced larger numbers of IFN-γ-secreting cells.

3.5. CTL responses

To confirm the primary $CD8^{+}$ cellular immune responses, mice were immunized with various DNA constructs as outlined above. At week 6, splenocytes from the mice in each group were harvested and pooled, and the bulk cultured CTL responses specific to HIV-1 Gag were measured following 6-day antigen restimulation in vitro. The target cells (p815) pulsed with MHC class I-restricted p24 peptide were incubated with a different ratio of effector cells. The vaccinated mice that received pcGM + Gag developed a higher level of HIV-1 Gag-specific lytic activity than those receiving pwt-GM + Gag and pGag alone (Fig. 5).

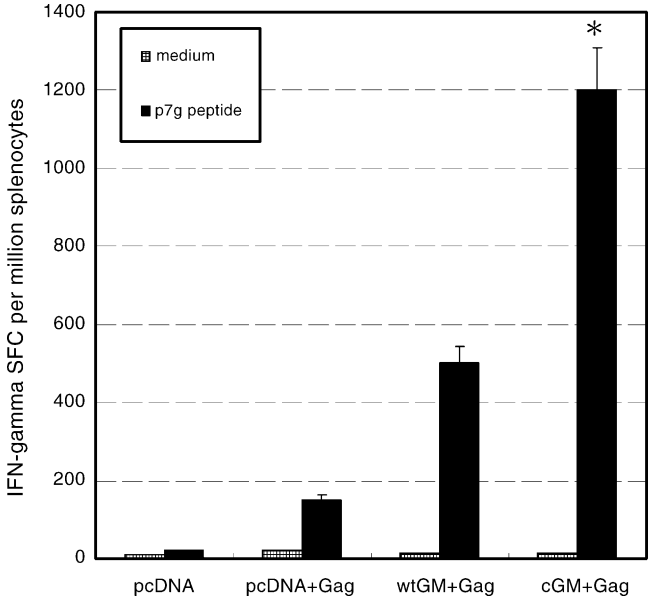

Fig. 4. ELISPOT assay. BALB/c mice were immunized as in Fig. 3. Anti-HIV-1 Gag CTL responses were measured at week 6 by ELISPOT assay. Splenocytes from the mice in each group were harvested and pooled, and the CTL responses specific to HIV-1 p24 peptide were measured following in vitro restimulation with p24 peptide (p7g, aa AMQMLKETI) for 24 h. The spot numbers are the means of the triplicates. Error bars indicate the standard deviations from triplicated cultures within the same plate. $^{*}P < 0.01$ by paired Student *t*-test. These data are representative of four independent experiments.

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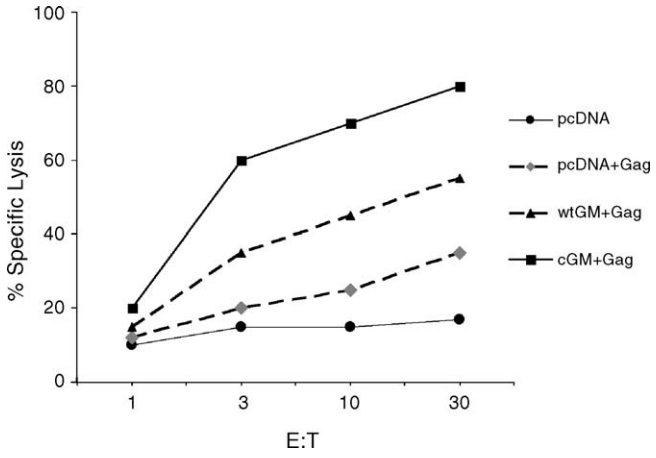

Fig. 5. Anti-HIV-1 Gag CTL responses. BALB/c mice were immunized as in Fig. 3. Anti-HIV-1 Gag CTL responses were measured at week 6. Splenocytes from the mice in each group were harvested and pooled, and the CTL responses specific to HIV-1 p24 peptide were measured following 6-day antigen restimulation in vitro. The target cells (p815) were pulsed with p24 peptide. These data are representative of four independent experiments.

3.6. Intracellular cytokine staining and flow cytometry analysis

Memory $CD8^+$ T lymphocytes are one of the most crucial components of antiviral effector cells. Therefore, we assessed the number of HIV-1 Gag-specific memory $CD8^+$ T cells expressing IFN-γ in the spleens of mice immunized with lower-dose DNA vector and challenged with vP1287. These cells were measured by brief stimulation in vitro for 24 h with MHC class I-restricted HIV p24 peptide (amino acid sequence: AMQMLKETI) followed by staining for $CD8^+$ and intracellular IFN-γ. We observed a high level of Gag-specific $CD8^+$ memory cells reactivated in the spleens of mice immunized with pcGM + Gag (1213 $CD8^+$/IFN-γ^+) within 4 days of vP1287 challenge (Fig. 6). In contrast, less than 0.01% of the total splenocytes were $CD8^+$ T cells expressing IFN-γ in control vector-immunized mice. The level of Gag-specific $CD8^+$ effector cells in the spleens of mice immunized with pwt-GM + Gag plasmids (564 $CD8^+$/IFN-γ^+) was lower than that obtained with pcGM + Gag. The background level in mice immunized with pcDNA3.1 and challenged with recombinant vaccinia virus expressing HIV-1 Gag was low, suggesting that Gag-specific $CD8^+$ IFN-γ-producing cells were generated from memory T cells. These studies confirm the functional significance of the enhanced cellular immunity afforded by plasmids encoding the codon-optimized murine GM-CSF gene.

3.7. Vaccinia virus titer in the ovaries of immunized mice

Finally, we addressed the question of whether or not the immune responses induced by HIV-1 Gag DNA vaccines adjuvanted with pcGM-CSF were protective. We also assessed the stability of memory in DNA vaccination by using recombinant vaccinia virus expressing HIV-1 Gag (vP1287). We chose the i.p. route for these experiments because this particular vaccinia virus has been found to replicate best in the ovaries [31]. On day 35 after the last DNA immunization, we challenged the immunized mice by i.p. infusion with vP1287. Four days after the challenge, the mice were sacrificed, their ovaries were removed, and the vP1287 titer in the ovaries was determined. Compared to the titer in control vector-immunized animals, mice immunized with the pcGM + Gag vaccine showed a 465-fold reduction in the average viral titer in the ovaries after an i.p. challenge with vP1287 (Fig. 7). In contrast, mice immunized with the pwt-GM + Gag vaccine showed a 115-fold reduction in viral titer in the ovaries and 54-fold reduction in pGag vaccine (Fig. 7). These results suggest that mice immunized with pcGM + Gag DNA vectors developed the best anti-Gag cellular immune responses which could protect the mice against infection with vaccinia virus expressing HIV-1 Gag.

4. Discussion

Several strategies have been investigated using GM-CSF proteins as an adjuvant [33]. Fusion proteins which comprise GM-CSF in tandem with a second protein have also been described for use as adjuvants [34,35]. An alternative approach to administering GM-CSF protein is gene therapy, wherein DNA encoding GM-CSF protein is administered and subsequently taken up by the recipient's cells and GM-CSF protein is produced in vivo. This approach offers the advantages of locally sustained release of the cytokine and has proven successful in murine models [36]. In addition, recipient cells can be removed from the organism, transfected with GM-CSF DNA, and then reintroduced into the organism at the desired location [37]. The expression levels observed from these approaches may be insufficient for providing the intended immunomodulatory adjuvant activity.

Recombinant forms of human GM-CSF have been created with the goal of improving the adjuvant activity or permitting large-scale protein production; however, these sequences alter the amino acid sequence and have been associated with adverse immunologic responses, presumably due to formation of novel epitopes that result from recombinant genes or exposed native protein backbone which is normally blocked by glycosylation [38]. For example, sargramostim differs from the native human GM-CSF by one amino acid at position 23 and a different carbohydrate moiety. In contrast, the present study provided for improved murine GM-CSF-encoding nucleic acid sequences that encode the identical amino acid sequence as the native gene and with improved in vivo adjuvant activity and in vitro expression level in all the cells tested. However, limited data are available here concerning the phenotype, the antigen-presenting capacity, and the number of antigen-presenting cells (APCs), activation level of APCs in injection sites after vaccination. Further study will be required to determine which of these factors is more significant.

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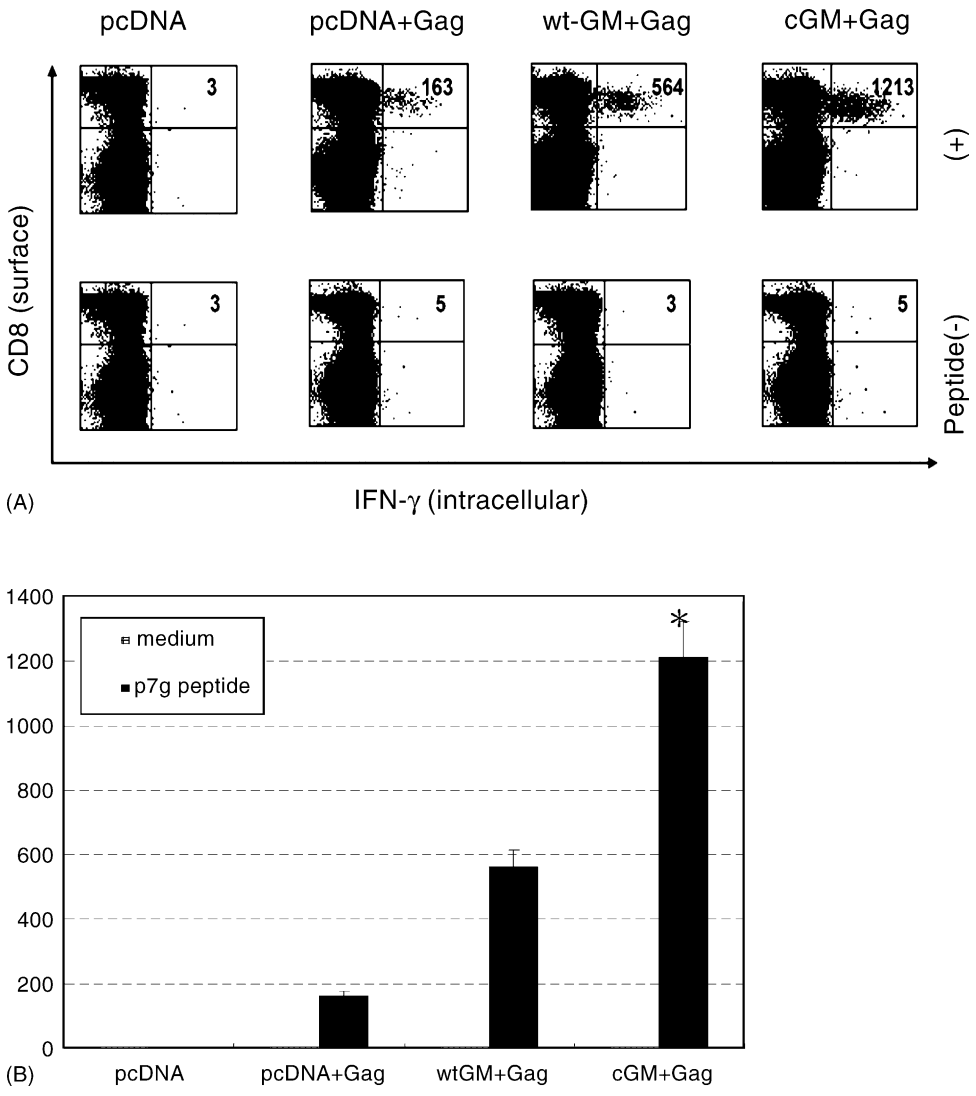

Fig. 6. Intracellular cytokine staining and flow cytometry analysis. BALB/c mice were i.m. injected two times with 50 μg of plasmid DNA at weeks 0 and 2. Thirty-five days after the last DNA injection, mice were i.p. challenged with a recombinant vaccinia virus vector encoding HIV-1 Gag (10^7 PFU per mouse). (A) Anti-HIV-1 Gag CTL responses were measured four days later. Splenocytes from vaccinia virus-challenged mice were cultured in vitro with p24 peptide (aa AMQMLKETI) for 24 h and were stained for both $CD8^+$ and intracellular IFN-γ. (B) The bar graph depicts the number of antigen-specific IFN-γ-secreting $CD8^+$ T-cell precursors/3×10^5 splenocytes (mean ± S.E.M.). The data presented in this figure are from one representative experiment of two performed.

The wild-type human and murine GM-CSF cDNA sequences were optimized by first identifying codons within the cDNA which were not associated with the codon usage in highly expressed genes in humans and mice, respectively. Each sub-optimal codon was replaced with those identified from highly expressed genes of the same species, which were known and available online, for example, at http://www.kazusa.or.jp/codon/. The novel GM-CSF sequences would improve the large-scale protein production from mammalian cells for research or clinical use.

Further, the codelivery of wild-type GM-CSF plasmids and DNA vaccines encoding malaria antigens [39] and other antigens [40–42] has led to the prediction of a greatly enhanced immune response characterized by a strong antibody, T helper and $CD8^+$ CTL immune priming. Furthermore, immunization with a bicistronic plasmid that coexpressed gp120 and GM-CSF under control of a single promoter led to a dramatic augmentation of vaccine-elicited $CD4^+$ T cell responses [16].

We have tested the enhancement of the primary and secondary immune responses primed with GM-CSF plasmid adjuvant. For these studies, we compared mice immunized with a plasmid encoding the HIV-1 gag gene with mice given the same plasmid in conjunction with GM-CSF plasmids. We first measured the ability of pcGM-CSF to expand the number of primed $CD8^+$ T-cells compared to pwtGM-CSF. Using the ELISPOT assay, we measured the number of antigen-specific T cells per million splenocytes and found that pcGM-CSF significantly increased the number of Ag-specific responders as measured by IFN-γ production. A similar pattern was observed in the antibody response of B cells measured by immunoblotting. In this case, the sera from mice that had

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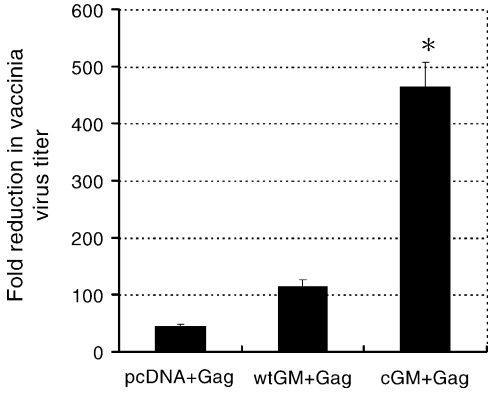

Fig. 7. Studies of vaccinia virus titer in the ovaries of immunized mice. Mice were immunized as outlined in the legend to Fig. 6 and were i.p. challenged with a recombinant vaccinia virus vector encoding HIV-1 Gag (10^7 PFU per mouse) at 35 days after the final DNA vaccination. Four days after the challenge, mice were sacrificed and ovaries were removed, homogenized, sonicated and pooled. The supernatants of the homogenates were assayed for virus titer on BSC-1 indicator cells. Results were expressed as fold of reduction in vaccinia virus titer in Gag-DNA-vaccinated mice versus the titer in control mice. $^*P < 0.01$ by paired Student *t*-test. The data presented in this figure are from one representative experiment of two performed.

received pcGM-CSF adjuvant produced a rapid generation of IgG antibody and the antibody titer to a much higher extent than sera from mice that had been given the antigen plasmids alone or combine with wild-type GM-CSF plasmids.

The enhanced CTL responses induced by the DNA vector containing pcGM + Gag molecules, compared to those induced by a DNA vector containing pwtGM + Gag molecules, also correlated with better protection against subsequent infection with a recombinant vaccinia virus expressing HIV-1 Gag in immunized mice. HIV-1 Gag CTL responses induced by the DNA vaccine may kill recombinant vaccinia virus-Gag-infected cells before more progeny viruses can be produced, therefore reducing the viral load in the ovaries of the mice. Mice immunized with DNA vector containing pcGM + Gag molecules showed a 465-fold reduction in recombinant vaccinia virus titer when compared to the results for mice immunized with the control DNA vector. On the other hand, mice immunized with a DNA vector containing pwtGM + Gag molecules showed only a 115-fold reduction in recombinant vaccinia virus titer compared to that for mice immunized with the control DNA vector.

The mechanism underlying the improvement of gene expression and induction of immune responses with codon-optimized, GM-CSF-adjuvanted DNA vaccination is still largely unclear. One explanation for this phenomenon is that some inhibitory sequences prevent efficient transport of these mRNAs from the nucleus to the cytoplasm [43,44]. Another possibility is that the inhibitory sequences result in rapid degradation of the mRNA in the nucleus and/or cytoplasm [6]. It has been shown that the use of codon-optimized HIV-1 sequences has resulted in significantly enhanced gene expression [6,43], but has rarely been studied in mammalian genes. It has been shown that the protein expression of native GM-CSF gene is poor and in a tissue-specific as well as an activation-dependent manner [21–23]. Not only codon-optimized GM-CSF gene increase the protein expression demonstrated in our study, but also the codon-optimized human IL-15 gene demonstrated similar result recently [10,45]. Further study is needed to investigate how generally true the effects of homologous and heterologous codon optimization in mammalian hosts are.

The competence of a vaccine to elicit high-frequency immune responses is critically dependent on the efficient presentation of antigen to T lymphocytes. This may be particularly important for DNA vaccines, which typically express low amounts of antigen that are largely restricted to the local site of immunization [46]. Clinical trials of DNA vaccines employ substantially lower injection doses and volumes than typical vaccine uses in mice after adjusting for body weight. Thus, it is likely that DNA vaccination in humans results in insufficient local inflammation, especially the cGMP-grade preparation, and recruitment of antigen-presenting cells to prime high-frequency immune responses. Co-administration of DC-specific cytokines such as GM-CSF and MIP-1α/Flt3L [11] may prove particularly useful in this scenario. Our novel codon-optimized GM-CSF gene significantly increased GM-CSF expression levels, which afforded potent adjuvant effects in mice immunized with limited vaccine doses and injection volumes. We had also generated codon-optimized human GM-CSF gene and tested with other vaccine genes (e.g. HPV-16 E7 and tumor genes). Although the practical utility of this strategy will require data from clinical trials, we predict that this molecular strategy to enhance immune responses will prove effective for both vaccines and other immunotherapeutic interventions [13,47,48].

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