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Intranasal administration of human immunodeficiency virus type-1 (HIV-1) DNA vaccine with interleukin-2 expression plasmid enhances cell-mediated immunity against HIV-1

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SUMMARY

DNA vaccine against human immunodeficiency virus type-1 (HIV-1) can induce substantial levels of HIV-1-specific humoral and cell-mediated immunity. To develop more potent HIV-1 DNA vaccine formulations, we used a murine model to explore the immunomodulatory effects of an interleukin-2 (IL-2) expression plasmid on an HIV-1 DNA vaccine following intranasal administration of the combination. When the vaccine and expression plasmid were incorporated into cationic liposomes and administered to mice, the HIV-1-specific delayed-type hypersensitivity response and cytotoxic T lymphocyte activity were significantly increased. Restimulated immune lymphoid cells showed enhanced production of both IL-2 and interferon-γ and reduced secretion of IL-4. The level of total antibody to HIV-1 antigen was not greatly changed by coadministration of the DNA vaccine and IL-2 expression plasmid. An analysis of serum HIV-1-specific IgG subclasses showed a significant drop in the IgG1/IgG2a ratio in the group that received the plasmid–vaccine combination. These results demonstrate that the IL-2 expression plasmid strongly enhances the HIV-1-specific immune response *via* activation of T helper type-1 cells.

INTRODUCTION

Recently, two vaccines against human immunodeficiency virus type-1 (HIV-1) have been developed by our group.[1,2] One contains a macromolecular polyvalent peptide,[1] and the other is a DNA vaccine constructed using HIV-1_{IIIB} *env* and *rev* expression plasmids.[2] Both vaccines induce HIV-1-specific humoral and cell-mediated immunity (CMI) following intramuscular immunization. Recent studies have revealed that CMI plays an important role in combating viral infections such as HIV-1,[3] hepatitis type C and malaria. Cytotoxic T lymphocytes (CTL) not only recognize processed viral fragments presented on the infected cell surface and destroy virus-infected cells, but also target all viral gene products that are expressed during viral replication. Targeting viral proteins through the development of specific CTL responses could aid in lowering the viral load by interfering with viral assembly.

It has been suggested that the profile of cytokines secreted by peripheral blood mononuclear cells in response to various stimuli is altered as HIV infection progresses toward acquired immune deficiency syndrome (AIDS).[4] The process is accompanied by a change in the ratio of T helper type-1 (Th1) cytokines [interleukin-2 (IL-2), IL-12 and interferon-γ (IFN-γ)] to the Th2 cytokines (IL-4, IL-5, IL-6 and IL-10). The initial T-helper dysfunction is a defective proliferation and a reduction in IL-2 secretion in response to recall antigens. To enhance HIV-1-specific CMI induced by DNA vaccination, we employed an IL-2 expression plasmid (BCMGNeo-mIL-2) as an immunological adjuvant for the DNA vaccine. IL-2 is capable of initiating proliferation of activated peripheral blood T cells and natural killer (NK) cells, and inducing production of cytolytic effector cells both *in vitro* and *in vivo*.[5] We therefore incorporated IL-2 expression plasmid into the vaccine formulation. The present study was designed to evaluate whether IL-2 expression plasmid enhances the HIV-1-specific immune response induced by DNA vaccination *via* the intranasal (i.n.) route.

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MATERIALS AND METHODS

Animals and plasmids

Six- to eight-week-old BALB/c female mice were purchased from Japan SLC. Inc., Shizuoka, Japan. The vaccine plasmids, pCMV160IIIB encoding HIV-1_{IIIB} *env* and pcREV encoding HIV-1 *rev* have been previously described.[2] Although this vaccine was designed to elicit a specific immune response to

HIV-1 envelope protein, the *rev* expression plasmid was included since the expression of HIV-1 *env* is dependent on *rev* coexpression.[6] The IL-2 expression plasmid, BCMGNeo-mIL-2,[7] was kindly donated by Dr H. Karasuyama, Department of Immunology, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan. The mock plasmids, pCMV-empty and BCMGNeo-empty, were constructed by removing gp160 cDNA and mIL-2 cDNA from their respective expression plasmids. Each group consisted of five or six mice.

Preparation of DC-Chol:DOPE liposomes and animal treatment

Detailed methods for preparation of cationic liposomes and i.n. immunization have been reported previously.[8,9] A mixture of 3β[N (N′, N′-dimethylaminoethane) carbamoyl] cholesterol (DC-Chol) and dioleoylphosphatidylethanolamine (DOPE) in chloroform was used for the cationic liposomes. Prior to i.n. administration, an appropriate amount of DNA (mock plasmid, DNA vaccine and/or IL-2 plasmid) in 0·15 mol phosphate-buffered saline (PBS), pH 7·2, was mixed with the above liposome solution at a volume ratio of 17:3. Mice were anesthetized with diethyl ether, and 30 μl of the liposome mixture containing 2 μg each of the DNA vaccine plasmids (pCMV160IIIB and pcREV; hereafter referred to as pCMV160IIIB/REV) and 1 or 10 μg of BCMGNeo-mIL-2 plasmid were dropped into a nostril. The mice were able to ingest these preparations simply by breathing. A single administration was used in the present study.

Sample collection

Sample immune sera and faeces were collected on days 14 and 28 after immunization. Sera were collected by retro-orbital puncture under anesthesia with diethyl ether and stored at 4° until use. Faecal pellets (100 mg) were suspended in 1 ml of PBS. After centrifugation at 17000 ***g***, the supernatants were collected and stored at −20° until use.

In vivo *injection of monoclonal antibodies (mAb)*

For the *in vivo* inhibition study, mice were intraperitoneally (i.p.) injected with 0·1 mg of mAb 2 days after immunization. S4B6 hybridoma (anti-IL-2 mAb) and 11B11 hybridoma (anti-IL-4 mAb) were provided by the American Type Culture Collection (ATCC), Rockville, MD.

Delayed-type hypersensitivity response (DTH)

The DTH response was assessed on day 14 after immunization using a footpad swelling method as previously described.[2] Briefly, 25 μl PBS containing 4 μg of an HIV-1_{IIIB} V3 peptide (RGPGPAFVTI) were injected into mouse footpads. Control mice were injected with the same dose of sperm whale myoglobin peptide, ALVEADVA. After 24 hr, the extent of footpad swelling was estimated as the difference in thickness in units of 10^{-2} mm between the preinjection and postinjection footpads.

CTL assay

Spleen cells and cells of regional lymph nodes were collected on day 21 after immunization.[2] Approximately 1×10^6 lymphoid cells from the immunized mice were restimulated *in vitro* with HIV-1 V3 peptide (RGPGRAFVTI)-pulsed syngeneic spleen cells. After culturing for 5 days, the cytotoxic activity of these spleen cells was measured by a 6-hr ^{51}Cr-release assay using V3 peptide-pulsed target cells. The target cells were prepared using the same HIV-1 V3 peptide-pulsed P815 cells (H-2^d). The percentage of specific ^{51}Cr release was calculated as $100 \times$ (experimental release − spontaneous release)/(maximum release − spontaneous release). Target cells incubated in medium alone and with medium plus 5% Triton-X-100 were used to determine spontaneous and maximum chromium release, respectively.

Enzyme-linked immunosorbent assay (ELISA)

The titres of serum IgG, IgG1, IgG2a and faecal IgA against HIV-1 were examined on days 14 and 28 after immunization using ELISA as described elsewhere.[2,10] Briefly, 96-well microtitre plates were coated with 5 μg/ml of HIV-1_{IIIB} V3 peptide (NNTRKSIRIQRGPGRAFVTIGKIGN)-multiple antigenic peptide (MAP) or a sperm whale myoglobin peptide, ALVEADVA. The wells were treated with PBS containing 1% bovine serum albumin (BSA) and were incubated for 1 hr at room temperature. They were then treated with 100 μl of 500-fold diluted mouse sera and incubated for an additional hour at 37°. The bound immunoglobulin was characterized using affinity-purified horseradish peroxidase (HRP)-labelled antimouse IgG, IgG1, or IgG2a (Organon Teknika, West Chester, PA). For the estimation of secretory IgA antibody against the HIV-1_{IIIB} V3 peptide, rabbit anti-rat secretory component (SC) antibody (kindly provided by Dr B. Underdown, McMaster University Medical Center, Hamilton, Ont., Canada) was also used. Antibody titres were reported as the reciprocal $\log_2$ value of the final detectable dilution which gave an optical density at 490 nm (OD_{490}) of $>0{\cdot}2$ OD units above the preimmune control.

For quantification of IL-2, IFN-γ and IL-4, spleens were harvested on day 21 after immunization. Immune spleen cells were collected and passed through nylon-wool column.[11] The T-cell-enriched fraction was treated with anti-CD8 mAb (anti-Lyt 2.2, ATCC TIB-210) plus rabbit complement. These cells were over 95% $CD4^+$ T cells by fluorescence-activated cell sorter (FACS) analysis. The purified $CD4^+$ T cells and freshly isolated splenic mononuclear cells were cultured in the presence of a V3 peptide. This peptide, RGPGRAFVTIGK, contains both a helper[12] and a CTL[13] epitope for HIV-1_{IIIB}. Culture media were collected 48 hr after initiating cell culture, and the cell-free supernatants were stored at −80° until use. Cytokine levels in these samples were measured with a commercial EIA kit (Cytoscreen®, Biosource, New Hampshire, MA) according to the manufacturer's instructions.

Data analysis

All values were expressed as means ± standard error (SEM). Statistical analysis of the experimental data and controls was conducted with one-way factorial analysis of variance. Significance was defined at $P<0{\cdot}05$ in statistical analyses.

RESULTS

DTH response and CTL activity

HIV-1-specific DTH was analysed using the footpad swelling response on day 14 after immunization. A single i.n. administration of the DNA vaccine enhanced the swelling response compared to the effect of the pCMV-empty plasmid, and

administration of the IL-2 expression plasmid alone also slightly induced a response (Table 1). Furthermore, administration of the DNA vaccine with 1 μg or 10 μg of the IL-2 expression plasmid significantly enhanced the swelling response compared to DNA vaccination alone; and 10 μg of the IL-2 expression plasmid was more effective than 1 μg. These responses were suppressed by i.p. injection of an anti-IL-2 mAb. To evaluate whether the swelling response was affected by the BCMGNeo-empty plasmid, we examined the response induced by DNA vaccination with or without this plasmid. A similar immune response was observed in mice administered and not administered the empty plasmid, suggesting that the adjuvant effect of the IL-2 expression plasmid was derived from IL-2 rather than from the mock plasmid.

HIV-1-specific CTL activity was explored on day 21 after immunization. As shown in Fig. 1, administration of the DNA vaccine alone or the IL-2 expression plasmid alone induced some level of CTL activity (Fig. 1). However, significantly enhanced activity was observed following i.n. administration in both groups. DNA vaccination with 10 μg of the IL-2 expression plasmid induced higher CTL activity than did 1 μg. CTL activities were slightly increased by administration of the DNA vaccine with the BCMGNeo-empty plasmid compared with administration of the DNA vaccine alone at an effector to target ratio of 80 (no statistical difference). This might have been due to the CpG motif in the empty vector.[14] In addition, this CTL response was greatly suppressed by injection of the anti-IL-2 mAb. These results indicate that the IL-2 expression plasmid greatly enhances the CTL activity induced by DNA vaccination, and that the effect of the IL-2 expression plasmid on the CMI response is dose-dependent.

HIV-1-specific serum IgG, IgG1, IgG2a and faecal secretory IgA responses

Levels of HIV-1-specific serum IgG, IgG1 and IgG2a antibodies, as well as that of faecal secretory IgA, were determined on days 14 and 28 after immunization. On day 14 after immunization, administration of the DNA vaccine and IL-2 expression plasmid combination slightly decreased titres of serum IgG and faecal secretory IgA antibodies compared with DNA vaccination alone (no statistical difference). However, i.n. administration of the vaccine with the expression plasmid significantly lowered the serum IgG1 antibody titre and increased the IgG2a antibody titre compared with administration of the DNA vaccine alone (Table 2). These modifications in the immune response were again suppressed by the anti-IL-2 mAb. Injection of anti-IL-4 mAb strongly suppressed the IgG1 level. Similar results were observed in the examination on day 28 after immunization. These results suggest that DNA vaccination with the IL-2 expression plasmid induces a Th1-dominant immune response.

Cytokine production by immune lymphoid cells

IL-2, IFN-γ and IL-4 in the culture media of restimulated bulk spleen cells harvested on day 21 after immunization were measured by ELISA. Compared with DNA vaccination alone, DNA vaccination with 1 μg of the IL-2 expression plasmid elicited a slightly higher production of IL-2 and IFN-γ and a significant drop in IL-4 synthesis; DNA vaccination with 10 μg of the IL-2 expression plasmid strongly increased the production of IL-2 and IFN-γ and decreased IL-4 synthesis (Fig. 2a,b). To confirm the above results, we examined cytokine secretion of purified immune $CD4^+$ T cells. DNA vaccination with 10 μg of the IL-2 expression plasmid also significantly enhanced production of IL-2 and IFN-γ and decreased IL-4 synthesis. These results demonstrate that DNA vaccination with the IL-2 expression plasmid increases production of Th1 cytokine and reduces Th2 cytokine synthesis compared with DNA vaccination alone, providing additional evidence that coadministration of the DNA vaccine and IL-2 expression plasmid can induce a Th1-dominant immune response.

DISCUSSION

Over the past several years, DNA vaccines have been intensively studied in animal models in terms of their ability

Table 1. Footpad swelling response of mice immunized with HIV-1 DNA vaccine and IL-2 expression plasmid

		Swelling response ($\times 10^{-2}$ mm)	
Immunogens	μg	IIIB V3 peptide	Myoglobin peptide
pCMV160IIIB/REV	2/2	11·1 ± 2·2*	1·7 ± 0·5
with BCMGNeo-mIL-2	1	14·2 ± 1·7*,**	2·9 ± 0·8
with BCMGNeo-mIL-2	10	16·7 ± 2·1*,**	3·3 ± 1·4
with BCMGNeo-mIL-2 + anti IL-2 mAb	10	5·0 ± 0·3**	2·2 ± 0·6
with BCMGNeo-mIL-2 + anti IL-4 mAb	10	16·8 ± 2·6**	2·9 ± 0·9
with BCMGNeo-empty	10	12·1 ± 1·9*	3·4 ± 1·2
pCMV-empty	2	2·3 ± 0·6	2·4 ± 0·8
BCMGNeo-mIL-2	10	4·7 ± 1·2	2 ± 0·6

pCMV160IIIB/REV with or without BCMGNeo-mIL-2 expression plasmid was intranasally administered to BALB/c mice. The footpad swelling assay was performed on day 14 after immunization. *Indicates a mean value significantly different from that obtained with pCMV-empty ($P < 0.05$). **Indicates a mean value significantly different from that obtained with pCMV160IIIB/REV ($P < 0.05$). Data are means of three separate experiments.

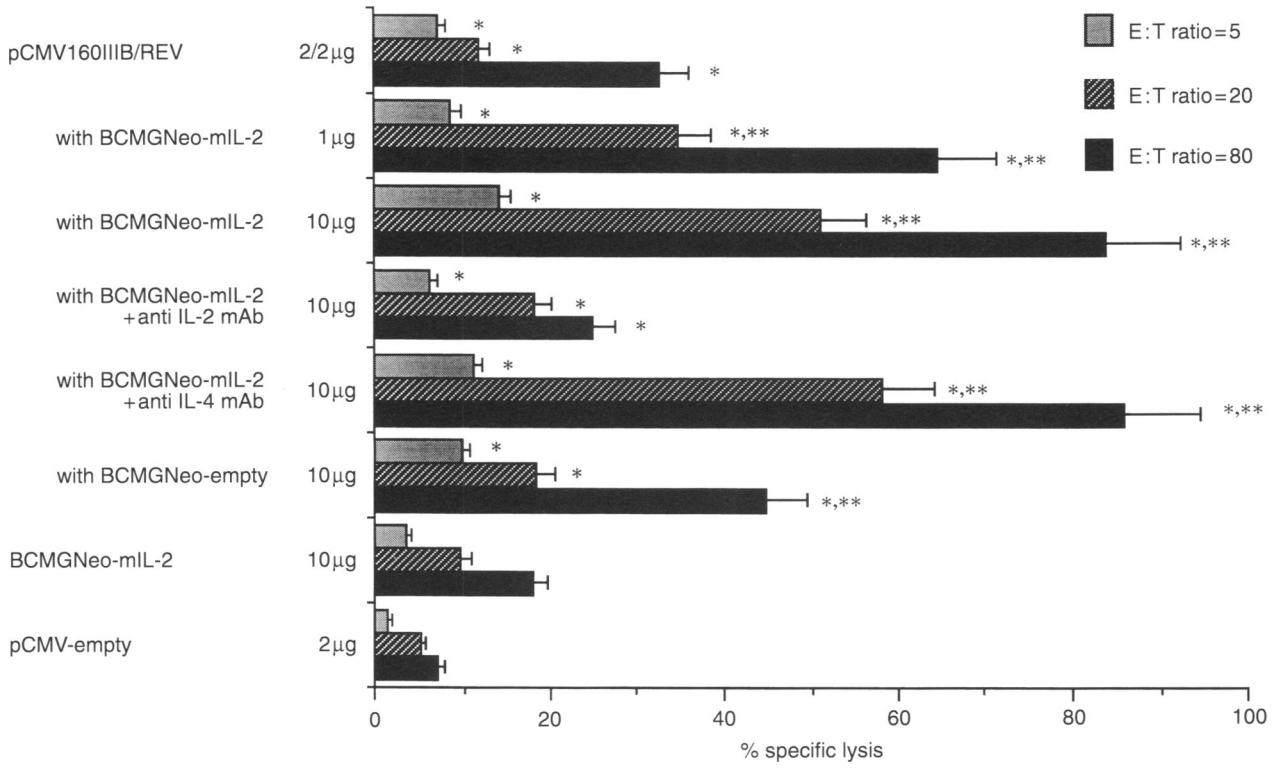

Figure 1. CTL response of mice immunized with HIV-1 DNA vaccine with or without IL-2 plasmid via i.n. administration, directed against V3 peptide-pulsed lymphoid cells. Immune lymphoid cells were restimulated *in vitro* with peptide-pulsed syngeneic mouse spleen cells. V3 peptide-pulsed P815 cells (H-2^d) were used as targets. The assay was performed at effector to target ratios of 5:1, 20:1 and 80:1. *Indicates a mean value significantly different from that obtained with pCMV-empty ($P<0{\cdot}05$); **Indicates a mean value significantly different from that obtained with pCMV160IIIB/REV ($P<0{\cdot}05$). Data are means of three separate experiments.

to provide protective immunity against a variety of diseases.[15] It has been suggested that the induction of a Th1-dependent HIV-1-specific CTL response is important in protecting against HIV infection and AIDS development.[3] Several studies have demonstrated that not only can CTL suppress HIV replication,[16] but it can also suppress the transcription of this virus[17] by enhancing the Th1 immune response. We previously developed an HIV-1 DNA vaccine capable of inducing both HIV-1-specific humoral and CMI responses in BALB/c mice, Std:JW/CSK rabbits and *Macaca fuscata* monkeys.[2] In the present study, we examined the effect of an IL-2 expression plasmid on a DNA vaccine. Our results showed that i.n. administration of the vaccine and expression plasmid combination greatly enhanced the HIV-1-specific CMI response compared with administration of the DNA vaccine alone, in terms of both DTH and CTL responses, and that enhancement of these responses was suppressed by anti-IL-2 mAb (Table 1; Fig. 1). DNA vaccination with the IL-2 expression plasmid also significantly lowered the serum IgG1/IgG2a ratio, enhanced production of IL-2 and IFN-γ, and reduced the level of IL-4 secreted by re-stimulated immune lymphoid cells (Table 2; Fig. 2a,b). These responses were also suppressed by the anti-IL-2 mAb. Our results demonstrate that the Th1 response is the predominant form of immunity when the DNA vaccine is administered i.n. with the IL-2 expression plasmid.

DNA vaccines provide the antigen required for protective immunization of the host without the need for a live organism or replicating vectors.[15] DNA vaccines are unlikely to be pathogenic or mutagenic to host cells since most plasmids exist in an unintegrated, circular form and do not replicate.[18] Because the immunogen is synthesized within the host by cells which have taken up the antigen-encoding DNA, *in vivo* protein synthesis allows for the processing, modification and presentation of the antigen to the immune system of this host in a manner similar to that which would occur during a natural infection. In addition, DNA vaccines are able to continue expressing their constituent genes for longer periods (up to several months[18]) compared to the shorter viability of recombinant protein (for example, the half-life of recombinant IL-12 in serum is 3·3 hr[19]). Our present results also suggested that these expression plasmids could strongly enhance a Th1-type immune response since they can consistently produce antigen for at least 1 week (data not shown).

Several routes have been used for the administration of DNA vaccines. Fynan *et al.*[15] compared the immune effectiveness of various administration routes (gene gun, intramuscular, intravenous, intranasal, intradermal, subcutaneous and intraperitoneal) using a DNA vaccine encoding an influenza viral haemagglutinin to stimulate the production of neutralizing antibodies in mice. After virus challenge, 95% survival was observed in mice immunized with the gene gun method and via the intramuscular route, and 83%, 76%, 75%, 67% and 0% survival was obtained with intravenous, intranasal,

Table 2. The titres of HIV-1-specific serum IgG, IgG1, IgG2a and faecal secretory IgA antibodies

Vaccine formulation	(μg)	Reciprocal $\log_2$ titre			
		Serum IgG	Serum IgG1	Serum IgG2a	Facal IgA
Day 14					
pCMV160IIIB/REV	2/2	6·2±0·5*	5·2±0·8*	2·1±0·3	7·8±0·3*
with BCMGNeo-mIL-2	1	6±0·5*	3·8±0·6*	4·5±0·6*,**	7·6±1·1*
with BCMGNeo-mIL-2	10	6±0·5*	2·2±0·4*,**	6·2±0·8*,**	7·6±1·1*
with BCMGNeo-mIL-2 +anti IL-2 mAb	10	7·2±0·4*	5·5±0·9*	3·2±0·8*	7·4±0·8*
with BCMGNeo-mIL-2 +anti IL-4 mAb	10	5·7±0·8*	2·4±0·4*,**	5·4±0·8*,**	6·8±0·9*
with BCMGNeo-empty	10	5·5±0·4*	4·9±0·6*	2·2±0·5	8±0·5*
pCMV-empty	2	<2	<2	<2	<2
BCMGNeo-mIL-2	10	<2	<2	<2	<2
Day 28					
pCMV160IIIB/REV	2/2	7·5±0·4*	5·8±0·2*	2·7±0·2	8·3±0·4*
with BCMGNeo-mIL-2	1	7·3±0·5*	4·2±0·2*	5·3±0·2*,**	8·2±0·7*
with BCMGNeo-mIL-2	10	6·8±0·4*	2·2±0·3*,**	6·8±0·2*,**	7·8±0·5*
with BCMGNeo-mIL-2 +anti IL-2 mAb	10	8·2±0·6*	6·3±0·4*	4·6±0·6*,**	9·2±0·6*
with BCMGNeo-mIL-2 +anti IL-4 mAb	10	4·6±1·2*	2·1±0·4*,**	4·3±0·9*,**	5·9±0·7*
with BCMGNeo-empty	10	7·8±0·3*	6·2±0·4*	3·1±0·3	8±0·5*
pCMV-empty	2	<2	<2	<2	<2
BCMGNeo-mIL-2	10	<2	<2	<2	<2

pCMV160IIIB/REV with or without BCMGNeo-mIL-2 expression plasmid was intranasally administered to BALB/c mice. On days 14 and 28 after immunization, the anti-HIV-1 antibody titres were determined by ELISA. Each titre is the reciprocal $\log_2$ value of the final detectable dilution which gave an optical density at 490 nm (OD_{490}) of 0·2 OD units above the preimmune control. A sperm whale myoglobin peptide (ALVEADVA) was used as a control peptide and 1·2±0·8 reciprocal $\log_2$ value was determined by ELISA. *Indicates a mean value significantly different from those of BCMGNeo-mIL-2 alone and pCMV-empty ($P<0·05$). **Indicates a mean value significantly different from pCMV160IIIB/REV($P<0·05$). Data are the mean of three separate experiments.

intradermal, subcutaneous and intraperitoneal immunization, respectively. We observed a strong Th1-dependent CMI response following i.n. administration of our DNA vaccine and IL-2 expression plasmid combination, and even noted a slight enhancement of immunity using only the empty vectors (Fig. 1). There are some reports in which a bacterial DNA plasmid containing the unmethylated CpG motif was able to enhance immune responses.[14] Therefore, the presence of this motif may form the basis of our observation.

HIV infection results in cytokine dysregulation,[20] including decreased IL-2 expression[21] and virus- and immune-mediated killing of CD4 lymphocytes. IL-2 is known to affect proliferation, differentiation and activation of T cells and of other lymphoid cell types, including (immature) thymocytes, B cells, lymphokine-activated killer and NK cells.[22] Even non-lymphoid cells, such as monocytes, oligodendrocytes and embryonic fibroblasts which express IL-2 receptors, are also responsive to IL-2. DNA vaccination with the IL-2 expression plasmid greatly enhanced IL-2 production (Fig. 2a), which may help in reducing the extent of HIV gp160-mediated T-lymphocyte apoptosis.[23] It may also help in overcoming an endogenous IL-2 deficit in patients with HIV infection as well as enhance the effects of other IL-2-responsive cells. Other studies have reported that coadministration of HIV-1 DNA vaccine and IL-2 expression plasmid strongly enhances HIV-1-specific IFN-γ production and suppresses IL-4 and IgG1 responses,[24] and induces a wider range of recognition with higher antibody titres against HIV-1.[25] Our results also showed that i.n. coadministration of the DNA vaccine and the IL-2 expression plasmid not only induced Th1 immune response against HIV-1 but provided high HIV-1-specific serum IgG and faecal IgA antibodies. Therefore, the coadministration may be useful in both protection against HIV-1 infection and treatment of AIDS and HIV disease. Based on the above, the IL-2 expression plasmid may represent one of the most important components of the ideal therapeutic vaccine.

Finally, we must consider whether administration of IL-2 expression plasmid would promote HIV replication in HIV-infected patients. Kinter *et al.* reported increased HIV replication in both recombinant IL-2 protein-stimulated $CD4^+$ T lymphocytes and mononuclear phagocytes derived from healthy HIV-seronegative individuals.[26] However, intermittent infusions of IL-2 in HIV-infected patients with baseline CD4 counts of more than 200 cells/mm^3 produced substantial and sustained increases in CD4 counts without increase in plasma HIV load, due to the induction by IL-2 of the potent suppressor activity of $CD8^+$ T cells.[27] Moreover, low doses of IL-2 therapy for HIV-positive individuals also enhanced immune function without toxicity.[28]

In summary, i.n. administration of DNA vaccine together with IL-2 expression plasmid can induce a strong Th1-dependent HIV-1-specific CMI response, which may be very important in controlling HIV infection and in slowing its progression to AIDS.[3]

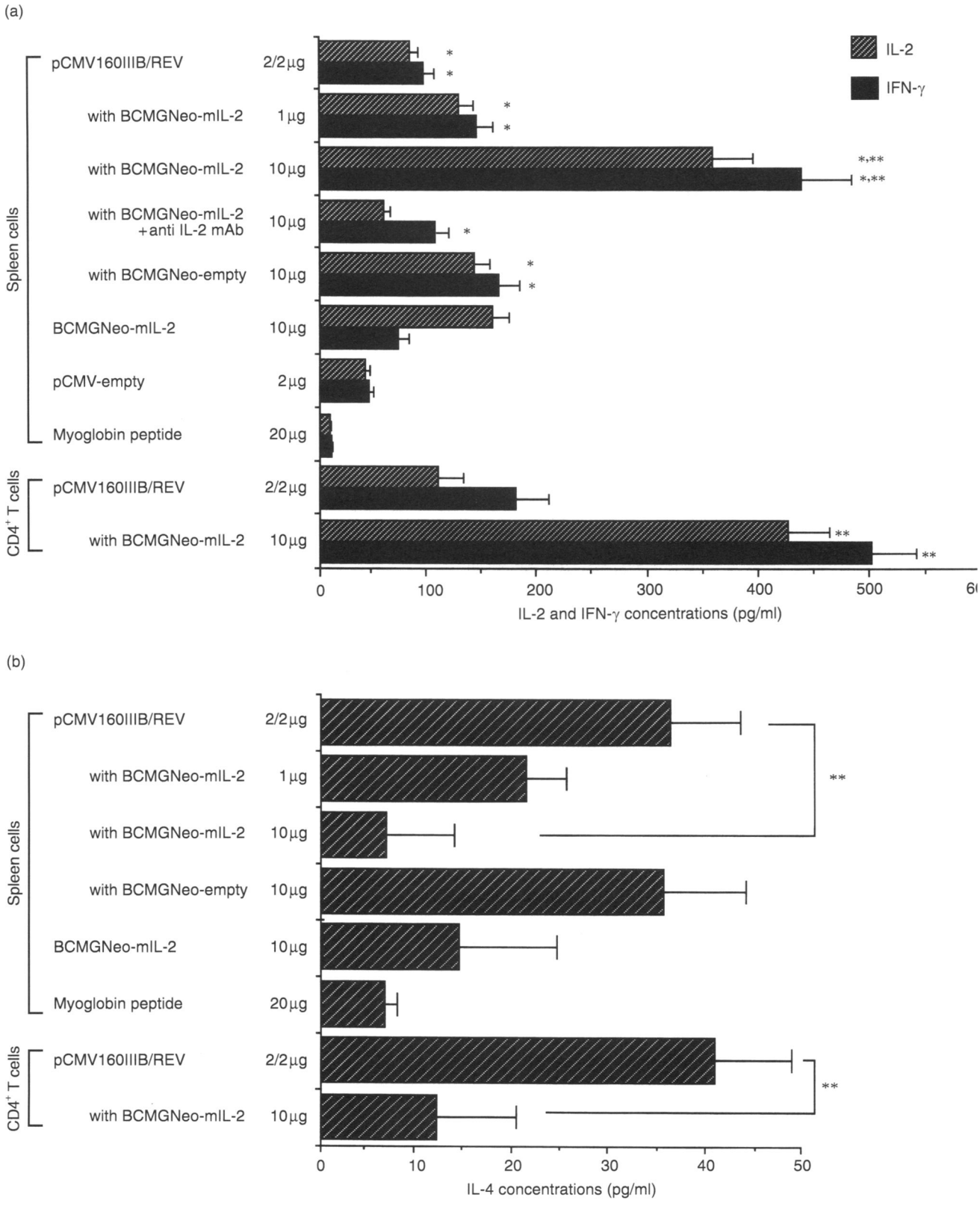

Figure 2. Cytokine concentrations of IL-2, IFN-γ (a) and IL-4 (b) in the culture media of splenocytes harvested from the immunized mice. BALB/c mice were intranasally administered HIV-1 DNA vaccine with or without IL-2 plasmid. The splenocytes were cultured in the presence of V3 peptide, and 48 hr later, cell-free supernatants were collected and subjected to a cytokine ELISA using appropriate assay kits. *Indicates a mean value significantly different from that obtained with pCMV-empty ($P<0{\cdot}05$); **Indicates a mean value significantly different from that obtained with pCMV160IIIB/REV ($P<0{\cdot}05$). Data are means of two separate experiments.

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