ASSOCIATION BETWEEN CIRCULATING FULL-LENGTH OSTEOPONTIN AND IFN-γ WITH DISEASE STATUS OF TUBERCULOSIS AND RESPONSE TO SUCCESSFUL TREATMENT

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Abstract. The T helper type 1 (Th1) immune response plays an important role in protective immunity, pathophysiology and development of tuberculosis (TB). To investigate whether osteopontin (OPN) and other Th1 response-related molecules are associated withTB disease status, including co-infection with HIV, and response to anti-TB treatment, circulating levels of full-length OPN (F-OPN), thrombin-cleaved N-terminal fragment of OPN (N-half OPN), IFN-γ, IP-10, IL-18, IL-12/IL-23 (p40), IL-10, IL-15 and C-reactive protein (CRP) were measured before and after anti-TB treatment. Patients with newly active pulmonary TB had significantly higher plasma levels of F-OPN, IFN-γ and CRP than healthy controls (HC). F-OPN, N-half OPN, IFN-γ, IP-10, IL-18 and IL-10 levels were higher in patients with extensive TB/HIV co-infection than in patients with a single disease of TB or HIV. Plasma levels of F-OPN correlated well with those of IP-10, IL-18 and N-half OPN among patients with active TB. The F-OPN, IFN-γ, IP-10 and CRP levels decreased significantly after effective anti-TB treatment. These data suggest that circulating OPN and Th1 response-related molecules, including IFN-γ, may be regulated in response to expansion of active TB and could serve as markers of disease activity before and during treatment.

Keywords: osteopontin, IFN-γ, CRP, tuberculosis, HIV/TB

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INTRODUCTION

Tuberculosis (TB) is one of the most important infectious causes of death worldwide (WHO, 2009). Despite its long historical interaction with humans, our understanding of host response to the TB

pathogen remains incomplete. Investigation of the molecular differences in host immune status between patients with active TB, co-infected with HIV and control subjects may provide a clue to understand the disease process.

In response to *M. tuberculosis*, activated macrophages and $CD4^+$ T lymphocytes produce Th1 cytokines, including IFN-γ, IL-12 and IL-18 (Schluger and Rom, 1998; van Crevel *et al*, 2002). IFN-γ triggers initiation of the major effector mechanism for the Th1 immune response (Flynn *et al*, 1993). IL-12 induction is observed following uptake of *M. tuberculosis* by dendritic cells and macrophages, which drives the production of IFN-γ in NK and T cells (van Crevel *et al*, 2002). Similarly, IL-18 exhibits strong IFN-γ inducing activity synergistically with IL-12 (Dinarello and Fantuzzi, 2003). The expression of IL-10 mRNA has been demonstrated in lymph nodes of TB patients, particularly in those with HIV/TB co-infection (Lin *et al*, 1996). Although IL-10 may down-regulate the immune response to mycobacterial infection (van Crevel *et al*, 2002), the exact role of IL-10 in TB remains controversial. IP-10, an IFN-γ inducible chemokine, is also predominant in active TB lymph nodes and the lung (Ferrero *et al*, 2003). Elevated circulating IP-10 levels have been reported in patients with active TB (Juffermans *et al*, 1999; Azzurri *et al*, 2005; Djoba Siawaya *et al*, 2009) and HIV/TB co-infection (Juffermans *et al*, 1999).

Osteopontin (OPN), a phosphorylated acidic glycoprotein associated with inflammation and tissue repair, is abundantly produced in the early stage of macrophage and T cell activation in granulomatous inflammation (O'Regan and Berman, 2000). OPN may polarize early Th1 cytokine responses through induction of IL-12 and suppression of IL-10 in macrophages (Ashkar *et al*, 2000). In a mouse model, a protective role of OPN in mycobacterial infection has clearly been demonstrated through experiments using OPN-null mice in which clearance of *M. bovis* BCG was reduced (Nau *et al*, 1999). In humans, OPN accumulates in well-formed granulomas with local mycobacterial infection, whereas OPN is absent or low in histologically ill-defined granulomas with disseminated infection (Nau *et al*, 2000). OPN is considered to play an active role in effective granuloma formation, inducing a Th1 response at an early stage of mycobacterial infection. Circulating OPN has also been measured in patients with active TB and their levels are generally high initially and decrease after anti-TB treatment (Koguchi *et al*, 2003; Inomata *et al*, 2005). Although these OPN levels have been reported to be correlated with Th1 cytokines, IFN-γ and IL-18 (Yamada *et al*, 2000; Inomata *et al*, 2005), the results of measuring circulating Th1 cytokine levels in human TB patients have often been inconsistent or unclear (Yamada *et al*, 2000; Morosini *et al*, 2003; Deveci *et al*, 2005; Inomata *et al*, 2005; Aktas *et al*, 2009). Immune reconstitution syndrome occurs after commencement of highly active antiretroviral therapy (HAART), at a stage when the *M. tuberculosis*-specific Th1 response is partially restored (Lawn *et al*, 2005). In HIV infected individuals, elevated OPN levels are found in cerebrospinal fluid and plasma and correlate with neurocognitive abnormalities (Burdo *et al*, 2008). OPN is the only pro-inflammatory cytokine found to increase after 1 month of HAART in lymph nodes (Li *et al*, 2004) and persists for 6 months of HAART (Chagan-Yasutan *et al*, 2009). OPN is susceptible to proteolytic fragmentation and a thrombin-cleaved N-terminal fragment of OPN (N-half OPN) is known to

affect its biological activity (O'Regan and Berman, 2000).

In this study, we attempted to address three questions unsolved by previous studies: 1) is OPN associated with TB even with HIV co-infection ($CD4^+$ T cell-depletion) in which granulomatous formation is generally poor? 2) is the N-half form, presumably cleaved by thrombin at the site of disease, more accurately connected with parameters of disease activity? 3) Do a variety of Th1-related molecules all coordinate with OPN levels? We investigated the concentrations of both full-length and N-half OPN, cytokines and a chemokine, including IFN-γ, IP-10, IL-18, IL-12/IL-23 (p40), IL-10 and IL-15, in the plasma of patients with newly active pulmonary TB, HIV/TB co-infection, HIV single infection and healthy controls and their levels within and between groups were compared. OPN and Th1 response-related molecules in patients with newly active pulmonary TB were also evaluated before and after anti-TB treatment. C-reactive protein (CRP) was simultaneously measured as a marker to monitor response to anti-TB treatment and an indicator of inflammation (Sahiratmadja *et al*, 2007; Peresi *et al*, 2008).

MATERIALS AND METHODS

Subjects

Twenty-three patients with pulmonary TB and 6 HIV/TB co-infected patients without highly active antiretroviral therapy (HAART) (HIV+TB+HAART) were recruited from the outpatient and inpatient clinics of Mae Chan and Chiang Rai hospitals, Chiang Rai Province, northern Thailand. HAART was defined as the regular use of two nucleoside reverse transcriptase inhibitors, NRTI [Stavudine (d4T) and Lamivudine (3TC)] plus a non-nucleoside reverse transcriptase inhibitor, NNRTI [Nevirapine (NVP) or Efavirenz (EFV)]. The patients with TB and HIV+TB+HAART-(HAART-) were all newly diaganosed pulmonary TB patients with sputum smears positive for acid-fast bacilli and confirmed by positive cultures for *M. tuberculosis* and abnormal chest radiographic findings. The patients had never received anti-TB treatment or had taken anti-TB drugs for less than 7 days at the time of enrollment. They had never received any immune-suppressive drugs or other immunomodulators. None of them had diabetes mellitus or other acute infections. On enrollment, the HIV/TB co-infected patients had not previously received antiretroviral therapy but were positive for HIV antibodies detected by particle agglutination assay (Serodia-HIV-1/2, Fujirebio, Tokyo, Japan) and/or immunochromatographic rapid test (Determine HIV-1/2, Abbott Laboratories, Abbott Park, Ill) followed by a confirmation test using enzyme-linked immunosorbent assay (ELISA) (Enzygnost Anti-HIV 1/2 plus ELISA, Dade Behring, Marburg, Germany).

Ten HIV patients not taking HAART (HIV+HAART-) and 17 HIV patients receiving HAART (HIV+HAART+) were recruited from the HIV Care and Treatment Project (Daycare clinic), Mae Chan Hospital. These patients had no previous history of TB. One patient who was HIV+HAART+ was taking isoniazid preventive therapy (IPT) for active TB on enrollment. Their sputum smears were negative for acid-fast bacilli and the cultures were negative for *M. tuberculosis*. They were negative (induration<5 mm) for tuberculin skin test and had no concomitant active AIDS-related opportunistic infections during the 30 days prior to enrollment. None had diabetes mellitus or

Table 1
Baseline characteristics of study subjects.

Characteristics	HC	TB	HIV+ HAART-	HIV+ HAART+	HIV+TB+ HAART-
	n=25	n=23	n=10	n=17	n=6
Age, median (range), years	35.0 (21-52)	46.0 (18-64)	37.5 (31-53)	39.0 (27-52)	43.0 (30-47)
Sex, number of males/females	15/10	15/8	6/4	8/9	5/1
WBC x 10^3, median	6.80	9.60	5.21	5.48	8.62
(range), cells/μl	(3.64-11.20)	(3.10-15.80)	(3.31-6.06)	(2.82-9.11)	(5.70-12.80)
$CD4^+$ T cell count, median	1,050	564	274	437	146
(range), cells/μl	(451-1,580)	(226-1,081)	(30-789)	(104-843)	(19-344)
≤200, No. (%)			3 (30.0)	4 (23.5)	4 (66.7)
201-500, No. (%)	1 (4.0)	10 (43.5)	5 (50.0)	7 (41.2)	2 (33.3)
>500, No. (%)	24 (96.0)	13 (56.5)	2 (20.0)	6 (35.3)	
CXR findings, No. (%)					
Normal	23 (92.0)		9 (90.0)	17 (100.0)	
Infiltrate /non-cavitary		20 (87.0)	1 (10.0)		5 (83.3)
Cavitary		3 (13.0)			1 (16.7)
No definite infiltration	2 (8.0)				
Site of TB infection by member					
Pulmonary		22			3
Extra-pulmonary					
Both		1			3

HAART, highly active antiretroviral therapy; HC, healthy control; TB, patients with tuberculosis; HIV+HAART-, HIV patients without HAART; HIV+HAART+, HIV patients with HAART; HIV+TB+HAART-, HIV/TB co-infected patients without HAART.

was receiving immune-suppressive drugs or other immunomodulators during the 90 days prior to enrollment.

Twenty-five Thai healthy controls (HC) were recruited through the blood bank at Mae Chan Hospital and served as controls. They had no previous history of TB or risk factors for TB. Their chest radiographs were normal. They had no latent TB infection detected by interferon-γ release assays [QuantiFERON®-TB Gold In-Tube (QFT), Cellestis, Victoria, Australia]. None of them had diabetes mellitus. All were negative for hepatitis B surface antigen, hepatitis C antigen and HIV antibodies using particle agglutination assay (Serodia-HIV-1/2, Fujirebio, Tokyo, Japan) and/or ELISA (Enzygnost Anti-HIV 1/2 plus ELISA, Dade Behring, Marburg, Germany).

The baseline characteristics of this patients and healthy controls are summarized in Table 1. Patients with TB had significantly higher white blood cell (WBC) counts (p<0.05) than HC; patients with HIV+TB+HAART- tended to have higher WBC counts. Patients with HIV+HAART- had significantly lower WBC counts than HC (p<0.01). The $CD4^+$ cell counts in TB patients were significantly higher than in HIV+HAART- patients (p<0.01), but were not significantly different from those with

Table 2
Clinical characteristics of six patients with HIV/TB co-infection.

Characteristics	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
Age in years/sex	42/Male	47/Male	37/Male	46/Female	30/Male	44/Male
CD4+ T cell count at TB diagnosis, cells/μl	46	198	19	321	94	344
CXR findings at TB diagnosis	Non-cavitary	Non-cavitary, infiltrates, pleural effusion	Non-cavitary, pleural effusion	Non-cavitary	Non-cavitary	Cavitary
Site of TB	PTB	PTB + EPTB (meningeal)	PTB	PTB + EPTB (colitis)	PTB + EPTB (lymphatic)	PTB
Treatment regimen for TB	2HRZE/4HR	2HRZE/4HR	2HEOS/18HE	2HRZE/4HR	2HRZE/4HR	2HRZE/4HR
HAART initiation during study period[a] (regimen)	Yes (d4T,3TC,NVP)	No	Yes (d4T,3TC,EFV)	No	No	No
Outcomes after 6-9 mo of anti-TB treatment	Cure	Cure	On treatment	Died[b]	Died[c]	Cure

PTB, pulmonary tuberculosis; EPTB, extrapulmonary tuberculosis; 2HRZE/4HR, treatment regimen consisted of the 2-months (mo) initial phase of isoniazid (H), rifampicin (R), pyrazinamide (Z) and ethambutol (E) followed by the 4-months continuation phase consisted of isoniazid and rifampicin; 2HEOS/18HE, treatment regimen consisted of the 2-months initial phase of isoniazid, ethambutol, ofloxacin (O) and streptomycin (S) followed by the 18-months continuation phase consisted of isoniazid and ethambutol; d4T, Stavudine; 3TC, Lamivudine; NVP, Nevirapine; EFV, Efavirenz; HAART, highly active antiretroviral therapy.
[a]HAART initiated 2 months after starting anti-TB treatment.
[b]After 87 days of anti-TB treatment.
[c]After 4 days of anti-TB treatment.

HIV+HAART+ (p=0.07). Among patients with HIV+HAART+, the median time interval between initiation of HAART and enrollment was 35 months (range 14-56 months). The baseline and follow-up characteristics of the 6 patients with HIV+TB+HAART- are shown in Table 2. Of these 6 patients, 3 had pulmonary TB and 3 had both pulmonary and extra-pulmonary TB, 2 of them died during anti-TB treatment with a principal diagnosis of disseminated TB. Among the remain patients, 3 were considered to be cured and 1 patient was still undergoing TB treatment after 6-9 months based on National Tuberculosis Program (NTP) guidelines. Of the 3 patients that could be followed-up, 1 patient with a baseline $CD4^+$ cell count<200 cells/μl had started HAART 2 months after anti-TB treatment. Twelve patients with TB and 3 patients with HIV+TB+HAART- were able to be followed-up after 6-9 months of anti-TB treatment and were considered as cured according to the standard criteria.

This study was approved by the Ethical Review Committee for Research on Human Subjects, Ministry of Public Health, Thailand (Reference number 15/2550) and the National Center for

Global Health and Medicine, Japan (Reference number 415). Written informed consent was obtained from all subjects prior to enrollment.

Blood samples

Blood samples were collected in ethylene diaminetetraacetic acid (EDTA) vacutainer tubes from patients and healthy controls at the time of enrollment and after 6-9 months of anti-TB treatment when they were considered as cured. After centrifugation at 1,000*g* for 10 minutes at room temperature, the plasmas were collected and kept at -80°C until used.

Determination of full-length and N-half OPN by ELISA

The levels for full-length (F-OPN) and N-terminal fragment OPN (N-half OPN) were determined with a sandwich ELISA kit according to the manufacturer's instructions (IBL, Gunma, Japan). The tests were done in duplicate and the concentrations of F-OPN/N-half OPN were calculated from a linear equation for each standard curve developed with recombinant human F-OPN/N-half OPN. The subtracted absorbance below zero was considered as zero. The lower detection limits of the F-OPN and N-half OPN assay kits were 3.3 ng/ml and 92.7 pg/ml, respectively.

Determination of cytokines, a chemokine and CRP

IFN-γ, IP-10, IL-18, IL-12/IL-23 (p40), IL-10 and IL-15 levels in plasma were determined using sandwich ELISA kits according to the manufacturer's instructions. The tests were done in duplicate and the concentrations of cytokines/chemokines were calculated from a linear equation for each standard curve. The subtracted absorbance below zero was considered as zero. The lower detection limits of the assays were 4.7 pg/ml for IFN-γ (BD Biosciences Pharmingen, San Diego, CA), 7.8 pg/ml for IP-10 (BD Biosciences Pharmingen), 12.5 pg/ml for IL-18 (MBL, Nagoya, Japan), 62.5 pg/ml for IL-12/IL-23 (p40) (BioLegend, San Diego, CA), 3.9 pg/ml for IL-10 (BioLegend) and 4.0 pg/ml for IL-15 (BioLegend).

Highly sensitive C-reactive protein (CRP) levels in plasma were measured by means of particle enhanced immunonephelometry using the BN system (*Cardio*Phase® *hs*CRP, Dade Behring, Newark, DE). The lower detection limit was 148 ng/ml. Values below this level were considered equal to 148 ng/ml. A level of 3,000 ng/ml in the serum was considered as the upper limit of normal.

Statistical analysis

Statistical analysis was performed using SPSS software version 17.0. The data were expressed as medians and ranges. Since not all the parameters exhibited normal distribution, comparison between two independent groups was performed using the nonparametric Mann-Whitney *U* test, and comparison between the two dependent groups was performed using the nonparametric Wilcoxon signed-ranks test. The correlations among the F-OPN, N-half OPN and T cell response-associated molecules were analyzed using a Spearman's rank correlation test. A p-value <0.05 was considered significant.

RESULTS

Circulating F-OPN levels in TB

The plasma F-OPN levels from patients with TB (251.9-959.9 ng/ml) and HIV+TB+HAART-(853.2-4,005.4 ng/ml) were significantly higher than in patients with HIV+HAART- (209.5-450.8 ng/ml) ($p<0.01$, $p<0.01$, respectively), HIV+HAART+ (141.2-655.1 ng/ml) ($p<0.01$, $p<0.001$, respectively) and HC

(37.3-517.8 ng/ml) ($p<0.000001$, $p<0.001$, respectively) (Fig 1a). The plasma F-OPN levels in patients with HIV+TB+HAART- were significantly higher than in patients with TB ($p<0.001$). Although the N-half OPN levels were below the detection sensitivity (92.7 pg/ml) in many study subjects (Fig 1b), the N-half OPN levels in patients with TB tended to be higher than in patients with HIV+HAART- and HIV+HAART+ and HC. Half of patients with HIV+TB+HAART- had even higher N-half OPN levels than patients with TB ($p<0.01$).

Changes in circulating IFN-γ, IP-10, IL-18, IL-12/IL-23 (p40), CRP and IL-10 in TB

Before anti-TB treatment, the plasma levels of IFN-γ, IP-10, IL-18, IL-12/IL-23 (p40) and CRP in patients with TB were significantly higher than in HC ($p<0.0000001$, $p<0.01$, $p<0.0001$, $p<0.00001$, and $p<0.0000001$, respectively), whereas IL-10 levels in patients with TB were significantly lower than in HC ($p<0.01$) (Fig 1c-1h). Patients with TB had significantly higher plasma IFN-γ, IP-10, IL-18 and CRP levels than patients with HIV+HAART+ ($p<0.001$, $p<0.0001$, $p<0.01$ and $p<0.000001$, respectively), and they had significantly higher IFN-γ and CRP levels than patients with HIV+HAART- ($p<0.01$ and $p<0.0001$, respectively).Patients with TB had significantly lower IL-12/IL-23 (p40) levels than patients with HIV+HAART- ($p<0.001$). Similarly, the plasma IFN-γ, IP-10, IL-18, IL-10 and CRP levels in patients with HIV+TB+HAART- were significantly higher than in HC ($p<0.01$, $p<0.001$, $p<0.001$, $p<0.01$ and $p<0.001$, respectively), patients with HIV+HAART- ($p<0.01$, $p<0.01$, $p<0.01$, $p<0.01$ and $p<0.01$, respectively) and patients with HIV+HAART+ ($p<0.01$, $p<0.001$, $p<0.01$, $p<0.01$ and $p<0.01$, respectively). The plasma IP-10, IL-18, IL-12/IL-23 (p40) and IL-10, but not IFN-γ and CRP levels in patients with HIV+TB+HAART- were significantly higher than in patients with TB ($p<0.001$, $p<0.01$, $p<0.05$ and $p<0.01$, respectively). The circulating levels of IL-15 were below the detection sensitivity of 4.0 pg/ml in almost all studied subjects, causing no significant differences (data not shown).

Correlations among circulating F-OPN, N-half OPN, IFN-γ, IP-10, IL-18, CRP and clinical parameters in tuberculosis cases

Correlations among plasma F-OPN, N-half OPN, IFN-γ, IP-10, IL-18, IL-12/IL-23 (p40), IL-10, IL-15 and CRP levels before anti-TB treatment were analyzed in patients with TB. Plasma F-OPN correlated significantly with N-half OPN ($r=0.508$, $p<0.05$), IP-10 ($r=0.500$, $p<0.05$) and IL-18 ($r=0.568$, $p<0.01$); whereas plasma F-OPN did not correlate with IFN-γ, IL-12/IL-23 (p40), IL-10, IL-15 or CRP. Positive correlations were also found between plasma levels of IP-10 and IFN-γ ($r=0.525$, $p<0.05$), IP-10 and IL-18 ($r=0.527$, $p<0.05$) and IL-18 and CRP ($r=0.519$, $p<0.05$). In patients with HIV+TB+HAART-, plasma F-OPN levels correlated significantly with IP-10 and IL-18 levels ($r=0.943$, $p<0.01$ and $r=0.829$, $p<0.05$, respectively).

The correlations between T cell response-associated molecules and the number of WBCs, lymphocytes, monocytes, $CD4^+$ T cells, $CD8^+$ T cells and $CD4^+/CD8^+$ ratio were analyzed in patients with TB. There were significant positive correlations between plasma F-OPN levels and WBC counts ($r=0.508$, $p<0.05$), CRP and WBC counts ($r=0.651$, $p<0.01$) and negative correlations between IFN-γ and $CD4^+/CD8^+$ ratios ($r=-0.474$, $p<0.05$), IP-10 and $CD4^+/CD8^+$ ratios ($r=-0.69$, $p<0.001$).

Circulating OPN, IFN-γ, IP-10 and CRP levels after anti-TB treatment

Plasma F-OPN, IFN-γ, IP-10, IL-18,

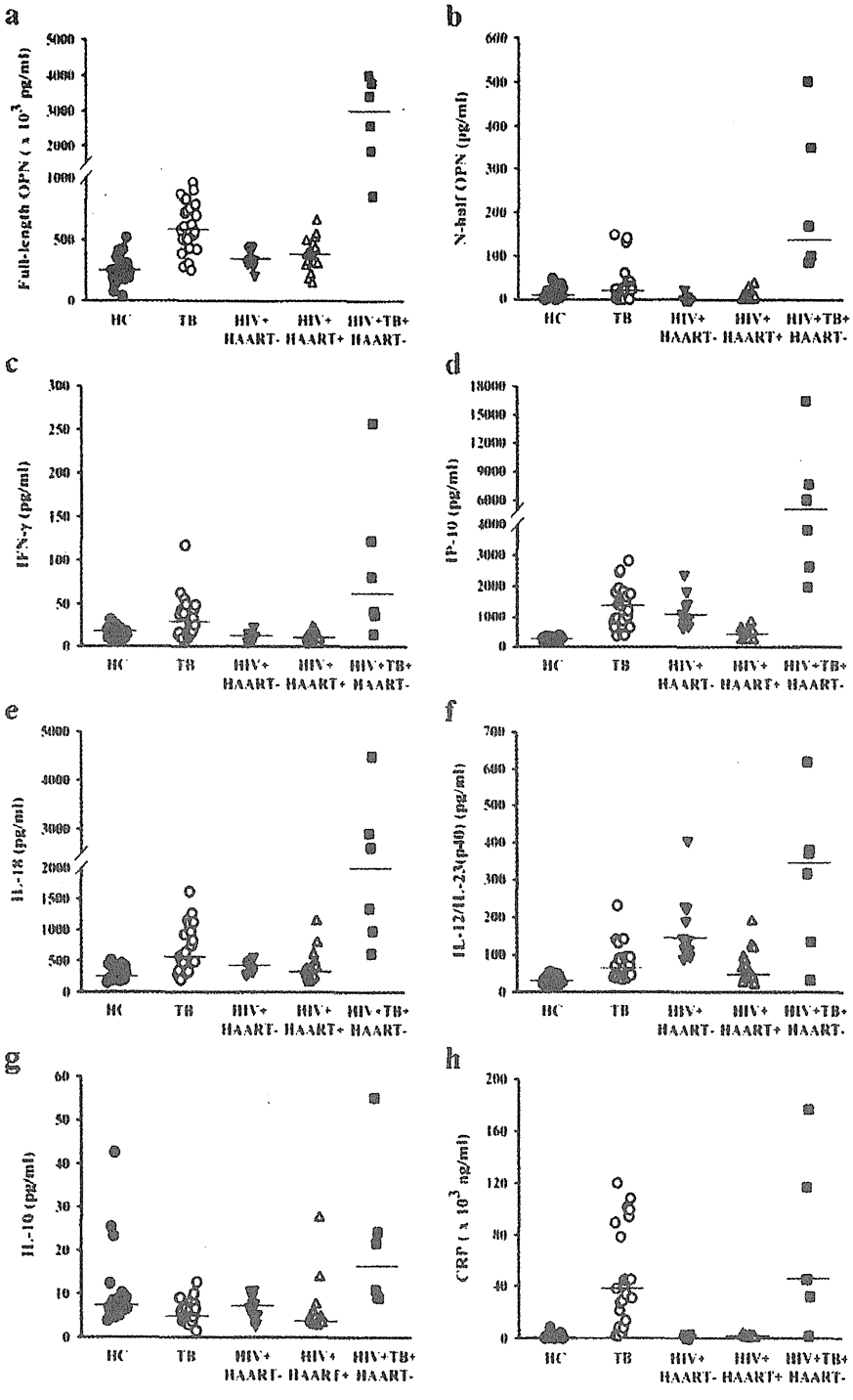

Fig 1–Circulating full-length OPN (a), N-half OPN (b), IFN-γ (c), IP-10 (d), IL-18 (e), IL-12/IL-23 (p40) (f), IL-10 (g) and CRP (h) levels in patients with tuberculosis (TB) and HIV/TB co-infection without HAART (HIV+TB+HAART-). HIV patients without HAART (HIV+HAART-) and with HAART (HIV+HAART+) were tested in comparison. Healthy individuals (HC) were used as controls. Bars represent the median values. The horizontal lines represent the lower limits of each measurement.

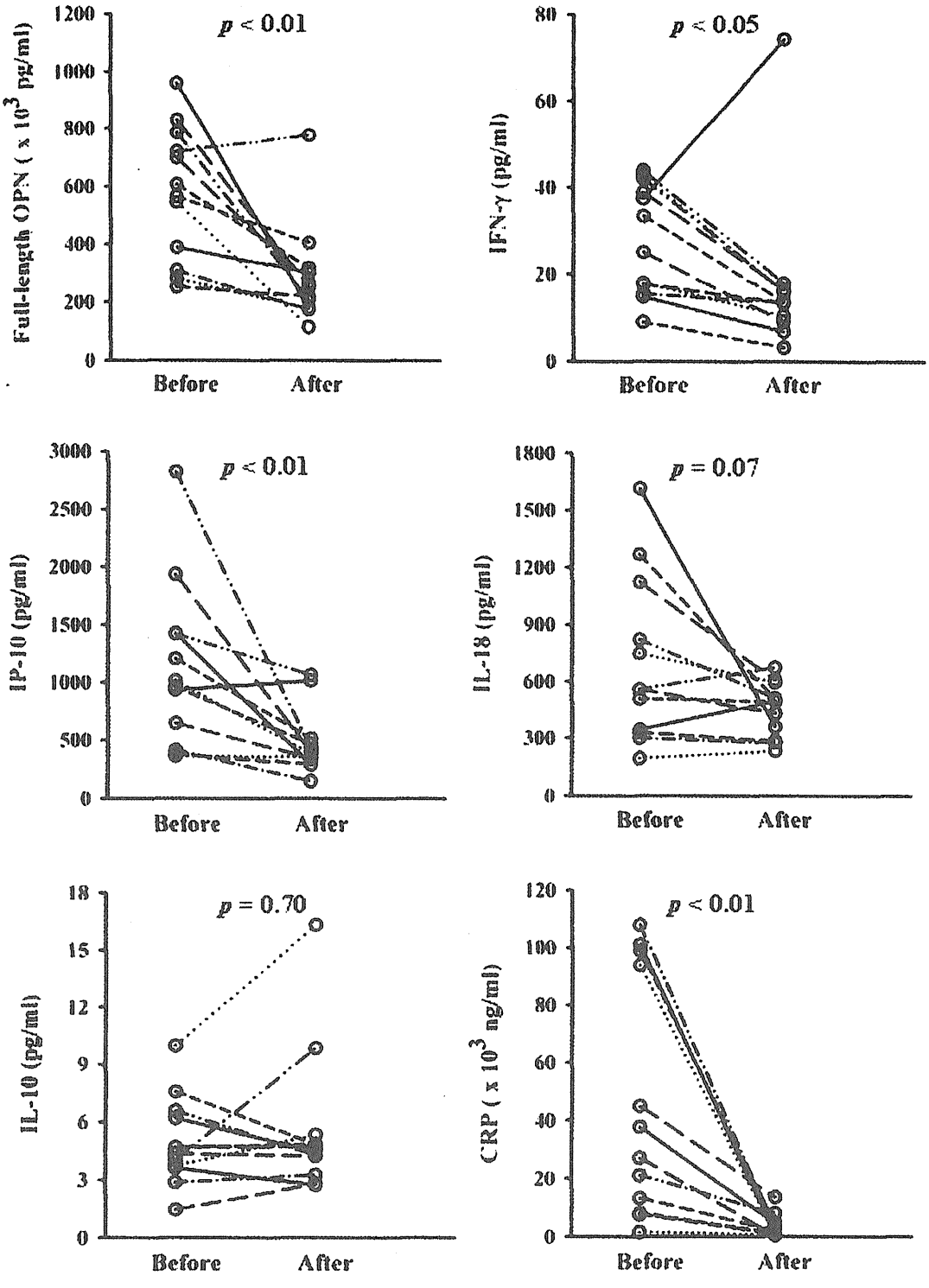

Fig 2–Circulating full-length OPN, IFN-γ, IP-10, IL-18, IL-10 and CRP levels among patients with active pulmonary TB before and after anti-TB treatment.

IL-12/IL-23 (p40), IL-10, IL-15 and CRP levels before and after 6-9 months of anti-TB treatment in the 12 patients with TB and in the 3 patients with HIV+TB+HAART- were evaluated. Significant decreases in plasma F-OPN, IFN-γ, IP-10 and CRP levels were seen in patients with TB after treatment ($p<0.01$, $p<0.05$, $p<0.01$ and $p<0.01$, respectively) (Fig 2). Although plasma IL-18 levels decreased in some TB patients after treatment, the change was not significant.

Plasma F-OPN, IFN-γ and CRP levels in patients with HIV+TB+HAART- tended to decrease after anti-TB treatment. After treatment, clinical improvement, negative sputum microscopy examinations and normal chest radiographs were observed.

DISCUSSION

To address the role of OPN in patients with TB, circulating F-OPN, N-half OPN and other cytokines and chemokine levels were evaluated along with clinical parameters in Thai patients with active pulmonary TB and HIV/TB co-infection. Circulating F-OPN, IFN-γ and CRP levels were significantly elevated in patients with active pulmonary TB and the levels decreased after effective anti-TB treatment. High concentrations of F-OPN, N-half OPN, IFN-γ, IP-10, IL-18 and IL-10 found in the plasma of patients with HIV/TB co-infection were unexpected, although this was a small-scale study. Levels of N-half OPN were much lower than those of F-OPN in all groups. Plasma levels of F-OPN correlated well with IP-10, IL-18 and N-half OPN levels among patients with active TB.

The high F-OPN levels in TB patients suggested a role for circulating F-OPN in disease activity among TB patients. Elevated circulating F-OPN levels in pulmonary TB patients is consistent with previous studies (Koguchi *et al*, 2003; Inomata *et al*, 2005). This may be partly due to leakage from granuloma sites evidenced by accumulation of OPN proteins in lung tissue sections from TB patients (Nau *et al*, 1997) and by abundant OPN expression in lymph nodes with well-formed granulomas (Nau *et al*, 2000). However, elevated circulating F-OPN and N-half OPN in patients with HIV/TB co-infection was not expected. HIV/TB co-infection is known to be associated with failure of granuloma formation and failure to control *M. tuberculosis* infection, thereby leading to mycobacterial dissemination (Corbett *et al*, 2003). The contribution of HIV infection to elevated circulating F-OPN is known and these levels correlate with HIV-induced CNS dysfunction, particularly in HIV-associated dementia, a severe neurocognitive abnormality that commonly occurs during the late stages of HIV infection (Burdo *et al*, 2008). Without receiving HAART, HIV infection chronically activates the host immune system to maintain a defense that only partially controls infection (Fauci, 1996), but chronic activation and replication, as well as storage of virus, leads to pathological consequences that may stimulate the production of various mediators of immune activation, including OPN. Collectively, prominent levels of circulating F-OPN in HIV/TB co-infection may not indicate disease status of effective granuloma formation but rather reflect spread of active TB lesions, large numbers of pathogens in the body or synergistic immune activation due to HIV/TB co-infection. F-OPN levels may not be equivalent to TB-associated inflammation simply measured by CRP because F-OPN levels did not correlate with CRP levels in the TB group.

The introduction of HAART among

HIV-infected patients usually results in the gradual reconstitution of the immune system (Weiss *et al*, 1999). HAART induced changes in the expression of many pro-inflammatory cytokines, including OPN in lymph nodes of HIV infected individuals 1 month after initiation (Li *et al*, 2004) but persistently elevated levels of circulating F-OPN during 6 months of HAART were observed (Chagan-Yasutan *et al*, 2009). In line with the latter findings, in this study, no differences in circulating F-OPN levels between HIV patients with or without HAART were found, despite a possible alteration in immune status with HAART. Different results are possibly due to differences in disease stage, regimen and duration of HAART.

Levels of circulating N-half OPN were much lower than those of F-OPN among all groups, and may not be helpful for monitoring disease activity. N-half OPN is generally more potent in causing cell migration and adhesions at the site of disease than in the uncleaved full-length form (Senger *et al*, 1994). In the synovial fluid of patients with rheumatoid arthritis (RA), N-half OPN has been detected at lower levels than F-OPN (Hasegawa *et al*, 2009). This indicates that N-half OPN exists at lower levels than its full form even at the site of inflammation. N-half OPN was detected in urine but not plasma from patients with RA at much lower levels than F-OPN (Shio *et al*, 2010). N-half OPN may not be stable in body fluids, including plasma, or is barely produced in tissues through strict regulation of thrombin/anti-thrombin balance. Thus, investigation regarding the functional form of OPN in TB and HIV/TB co-infection is further necessary when a more sensitive assay system is developed.

Elevation of circulating F-OPN, IFN-γ, IP-10 and IL-18 levels was documented in patients with active pulmonary TB. The results of circulating F-OPN, IFN-γ and IL-18 levels in patients with TB are consistent with other studies (Verbon *et al*, 1999; Morosini *et al*, 2003; Inomata *et al*, 2005). The finding of lower circulating IL-10 levels among TB patients than healthy controls is in contrast to some other studies (Verbon *et al*, 1999; Morosini *et al*, 2003; Deveci *et al*, 2005). This variability may result from a different status of healthy controls, in that all were negative on the interferon-gamma release assay (IGRA) in our study, whereas other studies consisted of controls with both positive and negative tuberculin skin tests (TST) (Morosini *et al*, 2003; Inomata *et al*, 2005). IL-10 levels in healthy controls in this study may have been affected by simultaneous infection with helminthes or tropical diseases, as is often seen in developing countries (Borkow and Bentwich, 2004). TB patients have different clinical characteristics, but only pulmonary TB patients with sputum smears positive for acid-fast bacilli (AFB) were recruited into this study, whereas another study included patients with both pulmonary and extra-pulmonary TB (Verbon *et al*, 1999).

The present results showed elevated IFN-γ and IP-10 levles were found in TB patients similar to previous studies (Juffermans *et al*, 1999; Azzurri *et al*, 2005; Djoba Siawaya *et al*, 2009). The present study demonstrated, for the first time, positive correlations between levels of F-OPN and IP-10, between IP-10 and IL-18 and between IP-10 and IFN-γ in patient with TB. Our findings of no correlations between circulating F-OPN and IFN-γ, between F-OPN and IL-12 and between IFN-γ and IL-12 are in contrast with some previous studies (Inomata *et al*, 2005; Pokkali and Das, 2009). Further studies are needed. OPN was found to be elevated

along other Th1-related molecules in patients with active TB.

In patients with TB, a significant decrease in circulating F-OPN, IFN-γ, IP-10, CRP levels and a trend toward a decrease in IL-18 levels were observed 6 to 9 months after anti-TB treatment. Furthermore, a decrease in circulating F-OPN, IFN-γ and CRP in 3 HIV/TB co-infected patients after completing treatment suggests these molecules may be useful for evaluating TB disease activity and monitoring response to treatment, as has been shown in previous studies (Koguchi *et al*, 2003; Inomata *et al*, 2005). However, discrepancies may occur (Verbon *et al*, 1999; Inomata *et al*, 2005; Djoba Siawaya *et al*, 2009) and caution is needed to interpret the results.

In conclusion, the present study confirmed the possible contribution of OPN for evaluating pulmonary TB disease activity, particularly in HIV/TB co-infected patients in association with Th1 response-related molecules. Clinically, the elevated OPN, IFN-γ and CRP levels and their decline after successful anti-TB treatment suggests circulating levels of F-OPN and Th1 response-related molecules, including IFN-γ, may be useful to determine expansion of active TB lesions and/or pathogens and may serve as markers of disease activity before and during treatment.

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Research Article

Novel Prophylactic Vaccine Using a Prime-Boost Method and Hemagglutinating Virus of Japan-Envelope against Tuberculosis

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Objective. Mycobacterium tuberculosis infection is a major global threat to human health. The only tuberculosis (TB) vaccine currently available is bacillus Calmette-Guérin (BCG), although it has no efficacy in adults. Therefore, the development of a novel vaccine against TB for adults is desired. *Method.* A novel TB vaccine expressing mycobacterial heat shock protein 65 (HSP65) and interleukin-12 (IL-12) delivered by the hemagglutinating virus of Japan- (HVJ)- envelope was evaluated against TB infection in mice. Bacterial load reductions and histopathological assessments were used to determine efficacy. *Results.* Vaccination by BCG prime with IgHSP65+murine IL-12/HVJ-envelope boost resulted in significant protective efficacy (>10,000-fold versus BCG alone) against TB infection in the lungs of mice. In addition to bacterial loads, significant protective efficacy was demonstrated by histopathological analysis of the lungs. Furthermore, the vaccine increased the number of T cells secreting IFN-γ. *Conclusion.* This vaccine showed extremely significant protection against TB in a mouse model, consistent with results from a similar paper on cynomolgus monkeys. The results suggest that further development of the vaccine for eventual testing in clinical trials may be warranted.

1. Introduction

Tuberculosis (TB) is a major global threat to human health, with about 2 million people dying every year from *Mycobacterium tuberculosis* infection. The only TB vaccine currently available is an attenuated strain of *Mycobacterium bovis*, bacillus Calmette-Guérin (BCG), although its efficacy against adult TB disease is unclear. Furthermore, multidrug-resistant TB (MDR-TB) and extremely drug-resistant TB (XDR-TB) are becoming big problems worldwide. For these reasons, a prophylactic and therapeutic vaccine against TB is sought. TB vaccines are classified into 4 main groups: (1) DNA vaccines, (2) recombinant BCG vaccines, (3) subunit vaccines, and (4) attenuated vaccines.

It is well established that protective immunity to *M. tuberculosis* depends on both $CD4^+$ and $CD8^+$ T cells [1–6]. DNA vaccines induce cellular immune responses, including the Th-1-type cellular immune response, and they prevent infections in animal models [7, 8]. In fact, several human clinical trials have recently been initiated to test the efficacy of DNA vaccines against emerging and re-emerging infectious diseases including hepatitis B [9], malaria [10–12], and HIV infections [13]. DNA vaccines have also shown their potential as TB vaccines in mouse

models [14–17]. However, in a guinea pig model, which is one of the most biologically relevant systems available for studying human pulmonary TB, DNA vaccines have not been proven more efficacious than BCG [18]. The efficacy of any experimental TB vaccine must be evaluated in human clinical trials, and a vaccine against TB is still anxiously awaited.

We have been developing a novel TB vaccine that is a DNA vaccine expressing mycobacterial heat shock protein 65 (HSP65) and interleukin-12 (IL-12), delivered by the hemagglutinating virus of Japan- (HVJ)- liposome or -envelope (HVJ-E) (HSP65 + IL-12/HVJ) [19–22]. The former vaccine was 100-fold more efficacious than BCG in a murine model on the basis of the elimination of *M. tuberculosis* [19]. In the present study, we demonstrated that the combination of BCG prime with HSP65 + IL-12/HVJ-E vaccine-boost was 10,000-fold more efficacious than BCG alone in a murine TB prophylactic model.

2. Materials and Methods

2.1. Bacteria. *M. tuberculosis* strains H37Rv and *M. bovis* BCG Tokyo, were kindly provided by Dr. I. Sugawara (JATA, Tokyo, Japan). *M. bovis* BCG Tokyo was maintained in synthetic Sauton's medium (Wako Chemicals, Osaka, Japan). For the mouse infection studies, a single colony of *M. tuberculosis* H37Rv was grown in Middlebrook 7H9 medium (DIFCO Laboratories, Detroit, MI; lot 137971 XA MD) supplemented with albumin-dextrose complex and grown at 37°C until approximately midlog phase. Aliquots were stored at −80°C and thawed 10 days before use. Each bacterium was grown to midlog phase in 7H9 medium.

2.2. Animals. Inbred and specific pathogen-free female BALB/c mice were purchased from Japan SLC (Shizuoka, Japan). Mice were maintained in isolator cages, manipulated in laminar flow hoods, and used between 8 and 10 weeks of age. All animal experiments were approved by the National Hospital Organization Kinki-chuo Chest Medical Center Animal Care and Use Committee. All vaccinations and experiments on isolated tissues were performed on anesthetized animals with sevoflurane. Infected animals were housed in individual microisolator cages in Biosafety Level (BL) 3 animal facility of the NHO Kinki-chuo Chest Medical Center.

2.3. Plasmid Construction. The *HSP65* gene was amplified from *M. tuberculosis* H37Rv genomic DNA, and cloned into pcDNA3.1 (+) (Invitrogen, San Diego, CA) to generate pcDNA-hsp65 (designated as HSP65 DNA) as described previously [19]. The *hsp65* gene was fused with mouse Igκ secretion signal sequence, and pcDNA-Ighsp65 (designated as IgHSP65 DNA) was generated. For construction of the mouse IL-12 (mIL-12) *p40* and *p35* single-chain genes, *mIL12p35* and *mIL12p40* genes were cloned from pcDNA-p40p35 [21], fused and cloned into pcDNA3.1 (+) to generate pcDNA-mIL12p40p35-F (designated as mIL-12 DNA).

2.4. HVJ-E Vaccination. HVJ-E was prepared as described previously (Figure 1) [19–23]. The HVJ-E complex was aliquoted and stored at −70°C until use. Groups of BALB/c mice were vaccinated 3 times at 3-week intervals with 100 μL of HVJ-E solution containing 50 μg of pcDNA-IgHSP65 and 50 μg of mIL12 DNA. These DNA vaccines were injected into both anterior muscles in the tibia. Mice were vaccinated using 1×10^6 colony-forming units (CFU) *M. bovis* BCG Tokyo by subcutaneous injection at 4 different sites (left-upper, right-upper, left-lower, and right-lower back). HVJ-E DNA vaccine containing pcDNA-IgHSP65 and -mIL12 DNA was designated as IgHSP65 + mIL-12/HVJ-E in this text.

2.5. Challenge Infection of Vaccinated Animals and Bacterial Load Determination. Mice were challenged by the intravenous route with 5×10^5 CFU of *M. tuberculosis* H37Rv 4 weeks after the third vaccination as described previously (Figure 2) [19, 24]. 0.2 mL of saline containing 5×10^5 CFU of H37Rv *Mycobacterium tuberculosis* were injected into tail vein of mice. At 5 and 10 weeks after *M. tuberculosis* H37Rv challenge, lungs, spleens, and livers were aseptically homogenized by using a homogenizer in saline, and serial dilutions of the organ homogenates were plated on 7H11 Middlebrook agar (Kyokuto. Tokyo, Japan). Plates were sealed and incubated at 37°C, and the number of colonies was counted 2 weeks later. Results were converted to $\log_{10}$ values. The $\log_{10}$ [mean ± standard deviation (S.D.)] values for CFU/organs/animals were calculated for each experimental group. Weight of lungs, liver, or spleen was measured by a balance (Sartorius Co. LP620S).

2.6. Histological Analysis. The lungs obtained from the mice were fixed with 10% buffered formalin and embedded in paraffin. Each block was sliced into 4-μm-thick sections and stained using hematoxylin and eosin. Semiquantitative morphometric analysis of pathological slides was performed by a method modified over that of Dascher et al. (2003) using a micrometer-attached microscope (Microphot-FXA, Nikon, Japan) [19, 25, 26]. The longer axis and minor axis of each granuloma in the field (×40 magnification) were measured and then multiplied and summed. Three random fields from each tissue section of mice were evaluated. The average score of the fields was designated as the granuloma index ($\times 10^{-2}$ mm^2). This method for the evaluation of granuloma area was significantly correlated with the granuloma area determined by a hematoxylin and eosin section scanning method.

2.7. ELISPOT Assay. The spleens were removed aseptically from vaccinated mice 3 weeks after the third vaccination. Antigen-specific IFN-γ-producing cells were determined by enzyme-linked immunosorbent spot (ELISPOT) as described previously [19]. Briefly, ELISPOT plates (MultiScreen IP Filtration plate MAIPS45; Millipore, Bedford, MA) were coated with antimouse IFN-γ MAb R4-6A2 (BD Biosciences Pharmingen. San Diego, CA). Spleen cells from vaccinated mice were suspended at 1×10^7 cells/mL (1×10^6 cells/well). The cells were placed into 6 antibody-coated wells, and rHSP65 protein (10 μg/mL) or PPD

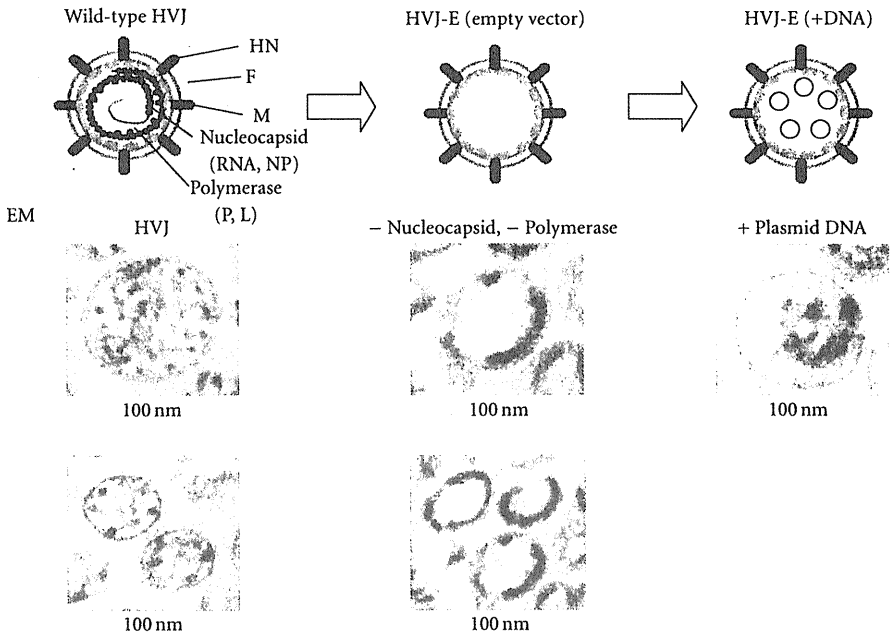

FIGURE 1: Hemagglutinating virus of Japan- (HVJ)- envelope vaccination: pcDNA3.1/HSP65DNA + IL-12DNA were incorporated into an HVJ-envelope empty vector (nonviral vector). Graphical representations of the HVJ-envelope empty vector in the presence or absence of DNA are shown. Electronic microscopy (EM) photographs of the HVJ-envelope empty vector are also shown.

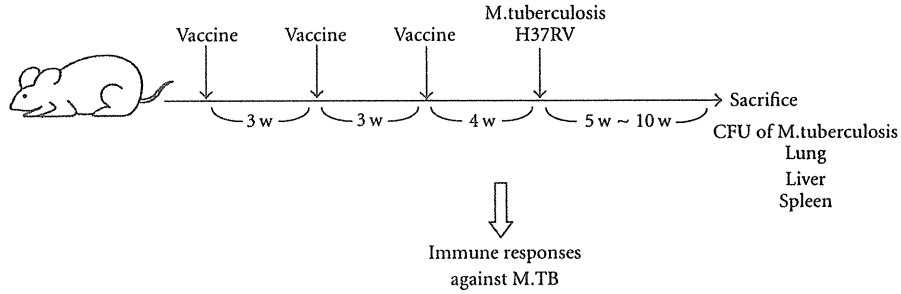

FIGURE 2: Groups of mice were vaccinated 3 times every 3 weeks using the prime-boost method and challenged intravenously with *M. tuberculosis* H37Rv as described in the Materials and Methods section. Five or 10 weeks after challenge with TB, protection was measured by enumerating bacterial loads (CFU) in the lungs, liver, and spleen of the vaccinated mice.

(10 μg/mL) was added to each well. After 20 h of incubation at 37°C, cells were removed by washing the plates, and the site of cytokine secretions was detected using biotinylated antimouse IFN-γ MAb XMG1.2 (BD Biosciences Pharmingen) and streptavidin-alkaline phosphatase conjugate (BD Biosciences Pharmingen). The enzyme reaction was developed with BCIP-NBT substrate (Vector Laboratories Inc., Burlingame, CA). Spot-forming cells (SFCs) were enumerated using the KS ELISPOT system (Carl Zeiss, Hallbergmoos, Germany).

2.8. Statistical Analysis. Dunnett's tests (multiple comparisons) were used to compare $\log_{10}$ values of CFUs between groups following challenge and used to compare T-cell responses between groups in the ELISPOT assay. A P-value of $< .05$ was considered significant.

3. Results and Discussion

3.1. Results

3.1.1. Prophylactic Efficacy Using Prime-Boost Method. The IgHSP65 + mIL-12/HVJ-E and BCG vaccines were administered using the prime-boost method as shown in Table 1.

At 5 and 10 weeks after intravenous challenge of *M. tuberculosis* H37Rv, the number of CFUs in the lungs, spleen, and liver were determined. Figure 3(a) shows the result of bacterial loads 5 weeks after challenge.

Table 1: BCG-HVJ-E/HSP65 DNA + IL-12 DNA Prime/Boost Experiment. Groups of mice were vaccinated 2 or 3 times with IgHSP65 + mIL-12/HVJ-E vaccine and/or BCG by using the prime-boost method. IgHSP65 + mIL-12/HVJ-E vaccine was injected intramuscularly, and BCG was injected subcutaneously. 4 weeks after the last immunization, *M. tuberculosis* H37Rv was challenged intravenously. 5 weeks and 10 weeks after TB challenge, protection was measured by enumerating bacterial loads (CFU) in the lungs, liver, and spleen from vaccinated mice. One week before the TB challenge, the immune responses of cytotoxic T cells, proliferation of T cells, and cytokines (IFN-γ, IL-2, IL-6) production were assayed.

Group	First immunization	Second immunization	Third immunization
1	—	—	—
2	—	—	BCG
3	HSP65 + IL-12/HVJ-E	HSP65 + IL-12/HVJ-E	HSP65 + IL-12/HVJ-E
4	BCG	HSP65 + IL-12/HVJ-E	HSP65 + IL-12/HVJ-E
5	HSP65 + IL-12/HVJ-E	HSP65 + IL-12/HVJ-E	BCG

13 mice per group.
3 mice for the *in vitro* assay prior to challenge (IFN-γ ELISPOT, etc.).
10 mice for the protection study (half of the mice were used for necropsy at 5 weeks after challenge and half at 10 weeks).

Vaccination by BCG prime + IgHSP65 + mIL-12/HVJ-E boost showed significant protective effects on the bacterial loads in the lungs as compared to BCG alone ($P < .01$). The prime-boost method using DNA and BCG vaccines showed extremely strong protective efficacy (>10,000-fold versus BCG alone) regardless of the order of administration (Figure 3(a)). Vaccination with BCG vaccine alone decreased TB CFUs in the lungs by 1 log unit as compared to nonvaccinated mice.

Vaccination with IgHSP65 + mIL-12/HVJ-E and BCG by the prime-boost method also showed significant protective efficacy on the bacterial loads in the liver as compared to BCG (>100-fold, $P < .05$; Figure 3(b)). The combination of 2 vaccines and administration by the prime-boost method also exerted a significant protective effect on the bacterial load in the spleen as compared to naive control group (10-fold higher, $P < .05$; Figure 3(c)).

Body weight of vaccinated mice was similar in all vaccinated groups. Tissue weights of spleens and livers in the prime-boost groups were lower than those of naive group (Figures 4 and 5).

We also confirmed the greater enhancement of protective effects in the BCG-DNA vaccine combination groups than those in the naive control group or BCG-alone group 10 weeks after challenge (data not shown). These results indicate that treatment using 2 vaccines by the prime-boost method was more effective than BCG alone.

3.1.2. Histological Analysis. In addition to the reduction of bacterial loads, the efficacies of each vaccine were assessed by histological analysis. The number and size of granulomatous lesions in the lungs were significantly lower and smaller, respectively, in the mice vaccinated by the BCG prime-DNA boost group than in the naive control mice and BCG control mice groups (Figure 6). Quantitative evaluation of the granulomatous lesions clearly showed that the BCG prime with IgHSP65 + mIL-12/HVJ-E boost significantly reduced the granuloma index in the lungs as compared to naive and BCG groups ($P < .05$; Figure 7). Thus, vaccination by the prime-boost method has the capability to reduce pulmonary lesions caused by *M. tuberculosis* infection.

3.1.3. Immunological Analysis. Furthermore, BCG prime with IgHSP65 + mIL-12/HVJ-E boost augmented the proliferation and IFN-γ production of HSP65 antigen-specific T cells in the K-S ELISPOT Assay. The efficacy of BCG prime with IgHSP65 + mIL-12/HVJ-E boost was higher compared with BCG Tokyo alone or IgHSP65 + mIL-12/HVJ-E prime with BCG boost (Figure 8).

These data indicate that the protective efficacies of BCG prime with IgHSP65 + mIL-12/HVJ-E boost are strongly associated with the number and activity of IFN-γ-secreting and HSP65-specific T cells. Taken together, combinational vaccination with BCG and IgHSP65 + mIL-12/HVJ-E by the prime-boost method is capable of augmenting T-cell activation. In addition, increase of IFN-γ-secreting cells is involved in the reduction of bacterial burden and lesions in the lungs. The efficacies of the prime-boost method are greater than those achieved by vaccination with BCG alone.

3.2. Discussion. In this study, we evaluated the protective efficacy of IgHSP65 + mIL-12/HVJ-E vaccine, using the prime-boost method. One of the significant findings was that the combination of IgHSP65 + mIL-12/HVJ-E and BCG led to a remarkably high degree of protection against intravenous challenge infection with virulent *M. tuberculosis*; bacterial numbers exponentially declined in 3 organs, especially in the lungs (10,000-fold lower than that of mice vaccinated with BCG alone; Figure 3(a)).

The pathological parameters of protection included reductions in the mean lung granulomatous lesion score in our study. The protective efficacies of BCG with IgHSP65 + mIL-12/HVJ-E administered by the prime-boost method were indicated on the basis of histopathological methods as well as bacterial loads. Histopathological analysis showed that mice vaccinated with BCG prime with IgHSP65 + mIL-12/HVJ-E boost had fewer and smaller lesions in the lungs and significantly less lung granuloma than naive mice and mice treated with BCG alone. These results suggest that severe toxicities (Koch phenomenon) were suppressed by the combination of two kinds of vaccines.

The data in the present study also show that the protective efficacy of BCG prime with IgHSP65 + mIL-12/HVJ-E boost is strongly associated with the emergence of IFN-γ-secreting T cells upon stimulation with HSP65. In the previous study, we demonstrated that *in vivo* function of CD8-positive T cells as well as CD4-positive T cells is involved in prophylactic efficacy of the IgHSP65 + mIL-12/HVJ-E in mice [22].

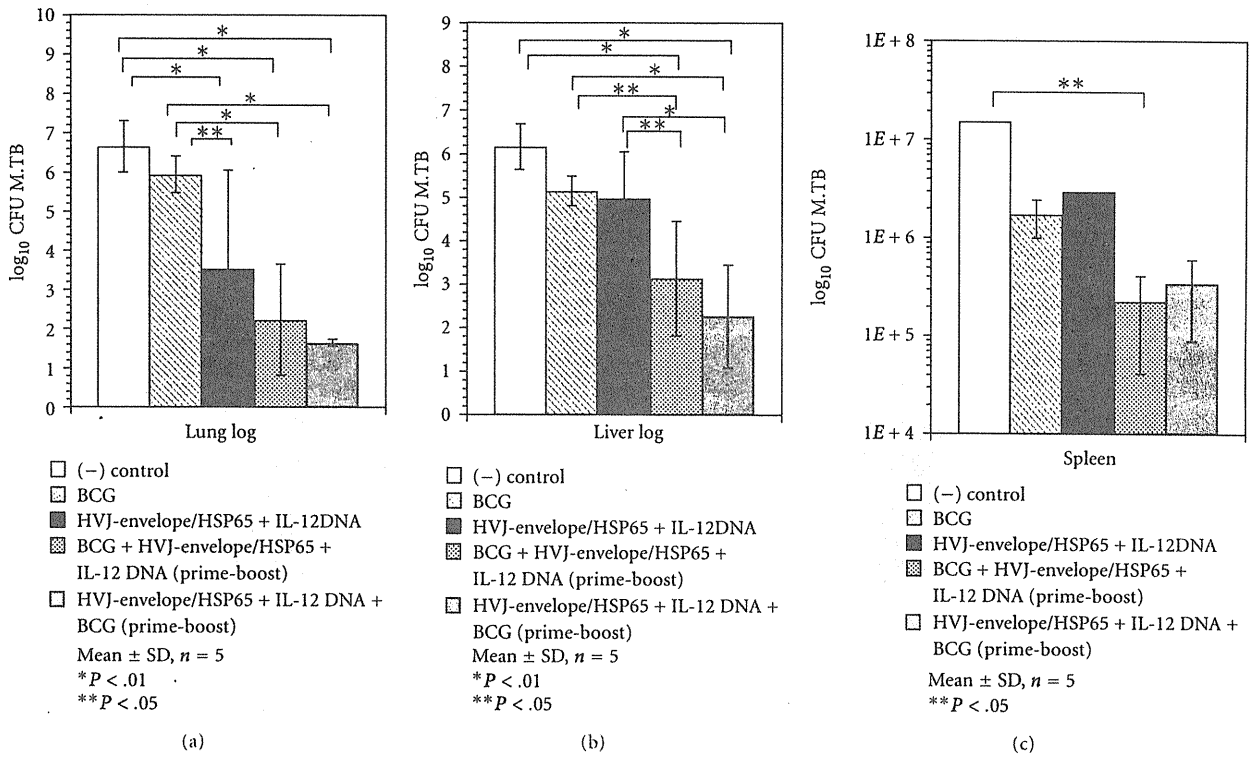

FIGURE 3: (a) Mouse protection studies using the prime-boost method. Groups of mice vaccinated with HVJ-envelope (HVJ-E) DNA and/or BCG were challenged by intravenous injection with *M. tuberculosis* H37Rv. Five weeks after challenge, protection was measured by enumerating the bacterial loads (CFU) in the lungs. Results are expressed as the mean $\log_{10}$ ± S.D. of CFU. The statistical significance of differences between individual groups in the CFU number was determined by Dunnett test ($n = 5$); $^*P < .01$ and $^{**}P < .05$; the statistical significance of differences ($P < .01$) of the G1 (naive) group compared to the G3 group (DNA/DNA/DNA), G4 group (BCG/DNA/DNA), or the G5 group (DNA/DNA/BCG). The statistical significance of differences ($^{**}P < .05$) of the G2 group (BCG-alone group) compared to the G3 group (DNA/DNA/DNA), that of differences ($P < .01$) of the G2 group compared to the G4 group (BCG/DNA/DNA), or the G5 group (DNA/DNA/BCG). (b) Mouse protection studies using the prime-boost method. Groups of mice vaccinated with HVJ-E DNA and/or BCG were challenged by intravenous injection with *M. tuberculosis* H37Rv. Five weeks after challenge, protection was measured by enumerating the bacterial loads (CFU) in the liver. Results are expressed as the mean $\log_{10}$ ± S.D. of CFU. The statistical significance of differences between individual groups in the CFU number was determined by Dunnett test ($n = 5$), $^*P < .01$; the statistical significance of differences ($P < .01$) of the G1 (naive) group compared to the G4 group (BCG/DNA/DNA), or the G5 group (DNA/DNA/BCG). The statistical significance of differences ($P < .05$) of the G2 group (BCG-alone group) compared to the G4 group (BCG/DNA/DNA). The statistical significance of differences($P < .01$) of the G2 group compared to the G5 group (DNA/DNA/BCG). The statistical significance of differences ($P < .05$) of the G3 group (DNA/DNA/DNA) compared to G4 (BCG/DNA/DNA). That of differences ($P < .01$) of the G3 group compared to the G5 group. (c) Mouse protection studies using the prime-boost method. Groups of mice vaccinated with HVJ-E DNA and/or BCG were challenged by intravenous injection with *M. tuberculosis* H37Rv. Five weeks after challenge, protection was measured by enumerating the bacterial loads (CFU) in the spleen. Results are expressed as the mean $\log_{10}$ ± S.D. of CFU. The statistical significance of differences between individual groups in the number of CFU was determined by Dunnett test ($n = 5$); $^{**}P < .05$; the statistical significance of differences ($P < .05$) of the G1 (naive) group compared to the G4 group (BCG/DNA/DNA).

In this study, we used the murine model of TB, which may not reflect the pathologic status of human TB. As to the difference of the infection route, our previous results in a guinea pig model used in a collaborative study with Dr. D. McMurray (Texas A&M University) showed that vaccination with HSP65 + guinea pig IL-12/HVJ resulted in better protection against pulmonary pathology caused by aerosol challenge with *M. tuberculosis* than BCG vaccination (data not shown).

In addition, we have recently confirmed that the prime-boost method was also effective in a cynomolgus monkey model [20–22]. We evaluated our HSP65 + human IL-12/HVJ (HSP65 + hIL-12/HVJ) in the monkey model infected by an intratracheal instillation (aerogenic route), which is currently the best animal model of human TB. Vaccination with HSP65 + hIL-12/HVJ resulted in better protective efficacy than that with BCG alone on the basis of the erythrocyte sedimentation rate test, chest X-ray findings, and immune responses. In addition, vaccination with HSP65 + hIL-12/HVJ resulted in increased survival for over a year. This was the first report of successful DNA vaccination against M. tuberculosis in a monkey model [21].

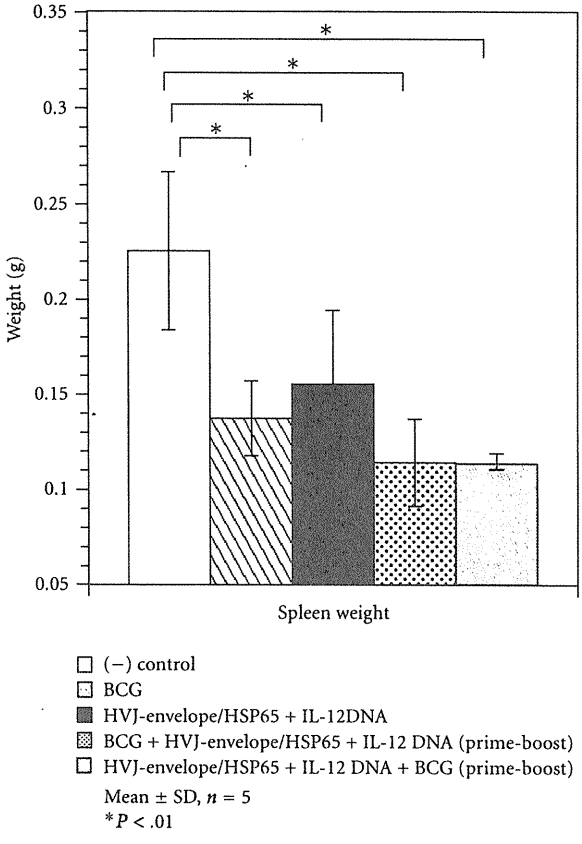

FIGURE 4: Tissue weight in mouse protection studies using the prime-boost method. Groups of mice vaccinated with HVJ-E DNA and/or BCG were challenged by intravenous injection with *M. tuberculosis* H37Rv. Five weeks after challenge, spleen weight was measured. Results are expressed as the mean ± S.D. in grams (g). The statistical significance of differences between individual groups in the weight was determined by Dunnett test ($n = 5$), $*P \leq .01$; the statistical significance of differences ($P < .01$) of the G1 (naive) group compared to the G2 group (BCG-alone group), G3 group (DNA/DNA/DNA), G4 group (BCG/DNA/DNA), or G5 group (DNA/DNA/BCG).

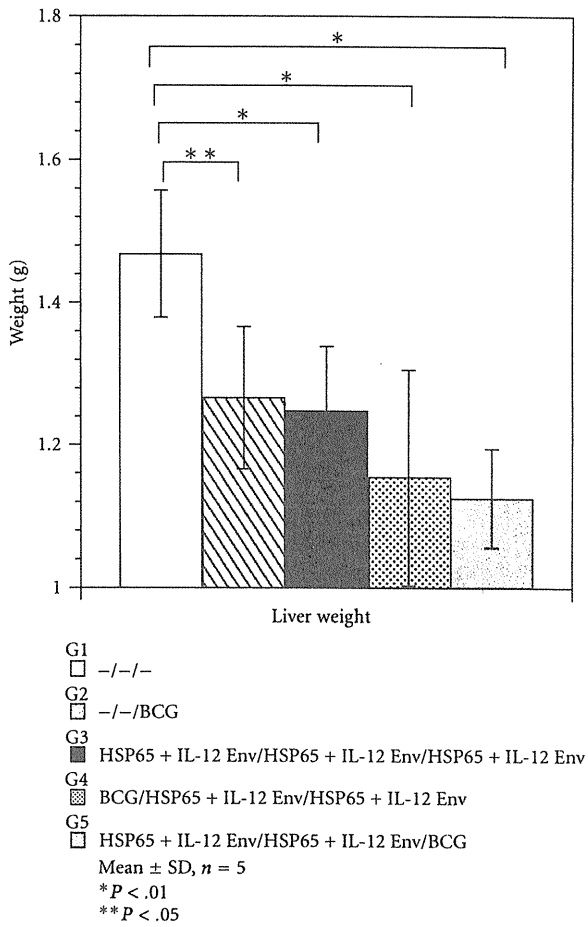

FIGURE 5: Tissue weight in mouse protection studies using the prime-boost method. Groups of mice vaccinated with HVJ-E DNA and/or BCG were challenged by intravenous injection with *M. tuberculosis* H37Rv. Five weeks after challenge, liver weight was measured. Results are expressed as the mean ± S.D. in grams (g). The statistical significance of differences between individual groups in the weight was determined by Dunnett test ($n = 5$), $*P < .01$; the statistical significance of differences ($P \leq .01$) of the G1 (naive) group compared to the G3 group (DNA/DNA/DNA), G4 group (BCG/DNA/DNA), or G5 group (DNA/DNA/BCG). $**P < .05$; that of differences ($P < .05$) of the G1 group compared to the G2 group (BCG alone group).

Most importantly, protective efficacy was augmented when BCG and HSP65 + hIL-12/HVJ were administered by the prime-boost method. Survival rates of BCG alone, saline control, HSP65 + hIL-12/HVJ-prime with BCG-boost, and BCG-prime with HSP65 + hIL-12/HVJ-boost groups were 33%(2/6), 50%(3/6), 50%(2/4), and 100%(4/4) at 12 months after the infection (aerogenic route), respectively [21]. We also evaluated immune responses in the monkey model of TB. Antigen-specific IFN-γ-production and proliferation of peripheral blood lymphocyte (PBL) were enhanced by the vaccination using the prime-boost method.

We also demonstrated efficacies in the monkey model when the boost was performed after a long-term period (4 months) from the prime. The prolongation of the survival was observed in the BCG-prime and HSP65 + IL-12/HVJ-booster group [27]. Improvement of ESR, increase of the body weight and augmentation of IFN-γ production, and proliferation of PBL were also observed in the BCG-prime and HSP65 + IL-12/HVJ-booster group.

Taken together, these results clearly demonstrated that BCG-prime with HSP65 + hIL-12/HVJ-boost could provide extremely strong protective efficacy against M. tuberculosis in a cynomolgus monkey model (intratracheal infection route), which is currently the best animal model of human TB [21].

The prime-boost method was reported in a study of the MVA85A vaccine, which is a modified vaccinia virus Ankara (MVA) strain expressing antigen 85A. In phase I studies in humans, this vaccine has induced high immune responses in previously BCG-vaccinated individuals [28].