

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

Chutharut Ridruechai^{1,2}, Shinsaku Sakurada², Hideki Yanai³, Norio Yamada³, Pacharee Kantipong⁴, Surachai Piyaworawong⁵, Panadda Dhepakson⁶, Srisin Khusmith¹ and Naoto Keicho²

¹Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand; ²Department of Respiratory Diseases, Research Institute, National Center for Global Health and Medicine, Shinjuku-ku, Tokyo, Japan; ³TB/HIV Research Project, Research Institute of Tuberculosis, Japan Anti-Tuberculosis Association, Chiang Rai; ⁴Department of Medicine, Chiang Rai Regional Hospital, Ministry of Public Health, Chiang Rai; ⁵Mae Chan District Hospital, Ministry of Public Health, Chiang Rai; ⁶Medical Biotechnology Center, National Institute of Health, Department of Medical Science, Ministry of Public Health, Nonthaburi, Thailand

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 with TB 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

Correspondence: Dr Srisin Khusmith, Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, 420/6 Ratchawithi Road, Bangkok 10400, Thailand. Tel: +66 (0) 2354 9100 ext 1594; Fax: +66 (0) 2643 5583
E-mail: tmskm@mahidol.ac.th

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 diagnosed 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 <i>n</i> =25	TB <i>n</i> =23	HIV+ HAART- <i>n</i> =10	HIV+ HAART+ <i>n</i> =17	HIV+TB+ HAART- <i>n</i> =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 $\times 10^3$, median (range), cells/ μ l	6.80 (3.64-11.20)	9.60 (3.10-15.80)	5.21 (3.31-6.06)	5.48 (2.82-9.11)	8.62 (5.70-12.80)
CD4 ⁺ T cell count, median (range), cells/ μ l	1,050 (451-1,580)	564 (226-1,081)	274 (30-789)	437 (104-843)	146 (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/ μ l	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.

^aHAART initiated 2 months after starting anti-TB treatment.

^bAfter 87 days of anti-TB treatment.

^cAfter 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 extrapulmonary 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/ μ l 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 1,000g 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 (*CardioPhase[®] hsCRP*, 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- ($p < 0.01$, $p < 0.01$, $p < 0.01$ 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,

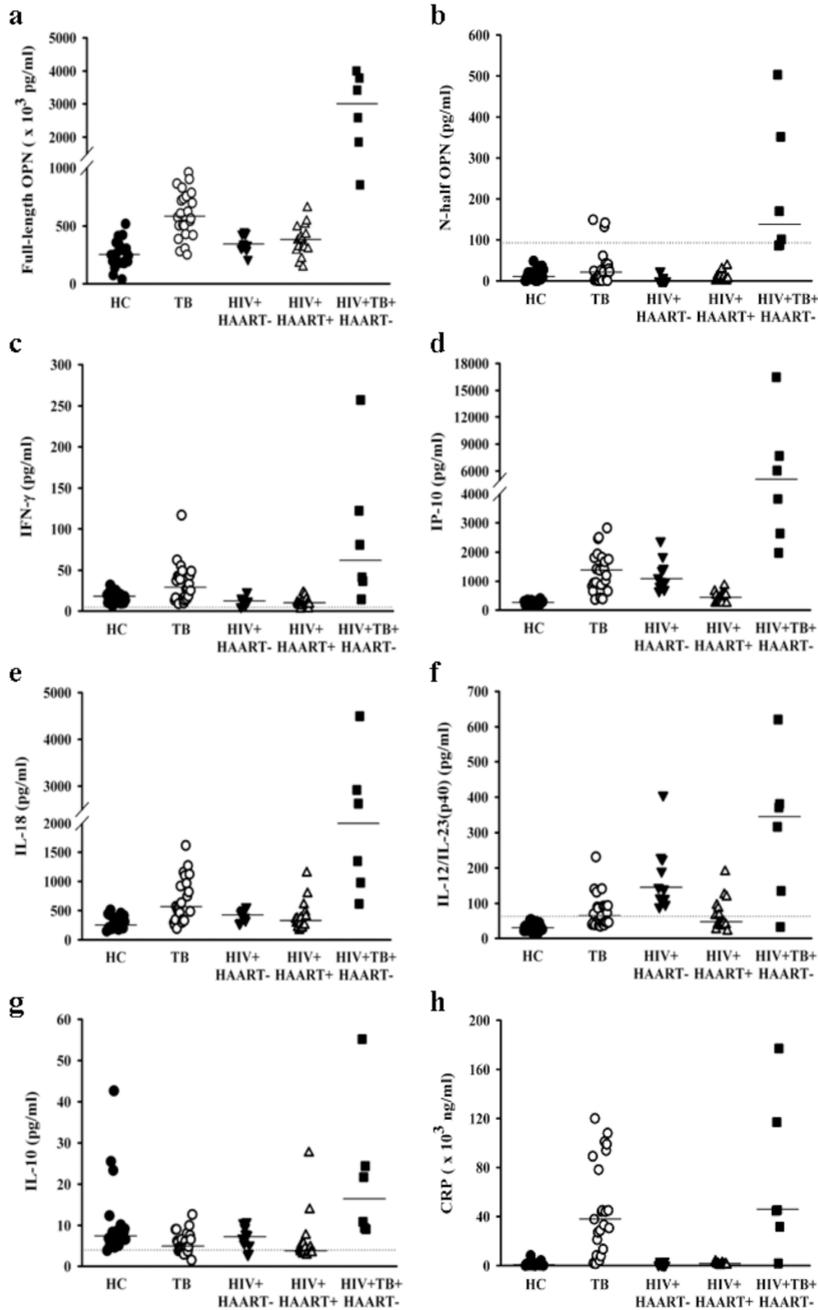


Fig 1–Circulating full-length OPN (a), N-half OPN (b), IFN- γ (c), IP-10 (d), IL-18 (e), IL-12/IL-23 (p40) (f), IL-10 (g) and CRP (h) levels in patients with tuberculosis (TB) and HIV/TB co-infection without HAART (HIV+TB+HAART-). HIV patients without HAART (HIV+HAART-) and with HAART (HIV+HAART+) were 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.

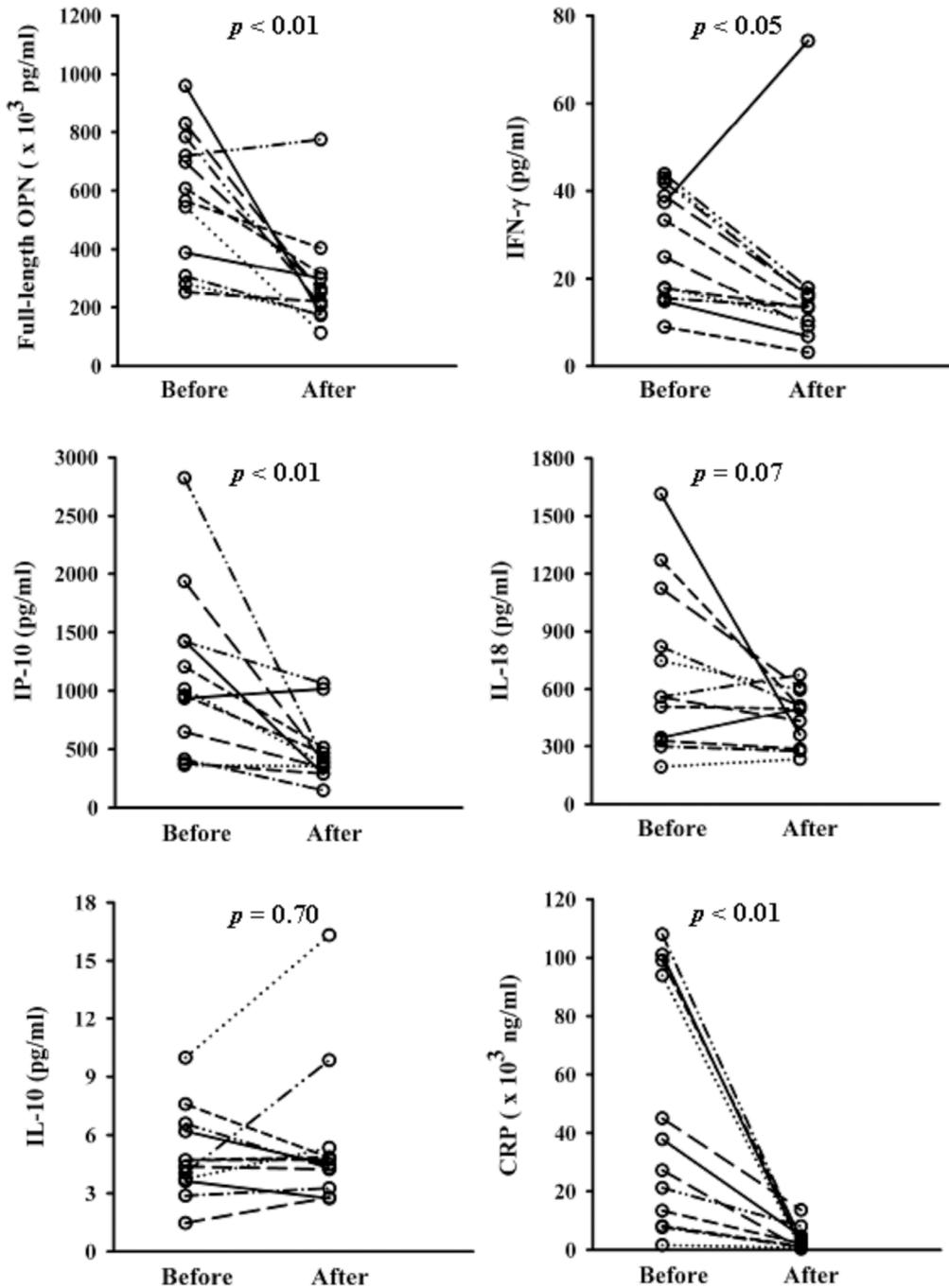


Fig 2—Circulating full-length OPN, IFN-γ, IP-10, IL-18, IL-10 and CRP levels among patients with active pulmonary TB before and after anti-TB treatment.

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 levels 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.

ACKNOWLEDGMENTS

We thank all the subjects for their kind participation in the study and the staff of the TB/HIV Research Project, Thailand, a collaborative research project between the Research Institute of Tuberculosis (RIT) and the Japan Anti-tuberculosis Association, and the Thai Ministry of Public Health for collecting the blood samples and obtaining the clinical information.

This study was supported by Health and Labor Science Research Grants for Research on Emerging and Re-emerging Infectious Diseases (H17-shinko-021 and H20-shinko-014), Ministry of Health, Labor and Welfare, Japan, Grants for Research on Global Health and Medicine (17C-1 and 20C-3), Ministry of Health, Labor and Welfare, Japan and Faculty of Tropical Medicine, Mahidol University, Thailand.

REFERENCES

- Aktas E, Ciftci F, Bilgic S, *et al*. Peripheral immune response in pulmonary tuberculosis. *Scand J Immunol* 2009; 70: 300-8.
- Ashkar S, Weber GF, Panoutsakopoulou V, *et al*. Eta-1 (osteopontin): an early component of type-1 (cell-mediated) immunity. *Science* 2000; 287: 860-4.
- Azzurri A, Sow OY, Amedei A, *et al*. IFN- γ -inducible protein 10 and pentraxin 3 plasma levels are tools for monitoring inflammation and disease activity in *Mycobacterium tuberculosis* infection. *Microbes Infect* 2005; 7: 1-8.
- Borkow G, Bentwich Z. Chronic immune activation associated with chronic helminthic and human immunodeficiency virus infections: role of hyporesponsiveness and anergy. *Clin Microbiol Rev* 2004; 17: 1012-30.
- Burdo TH, Ellis RJ, Fox HS. Osteopontin is increased in HIV-associated dementia. *J Infect Dis* 2008; 198: 715-22.
- Chagan-Yasutan H, Saitoh H, Ashino Y, *et al*. Persistent elevation of plasma osteopontin levels in HIV patients despite highly active antiretroviral therapy. *Tohoku J Exp Med* 2009; 218: 285-92.
- Corbett EL, Watt CJ, Walker N, *et al*. The growing burden of tuberculosis: global trends and interactions with the HIV epidemic. *Arch Intern Med* 2003; 163: 1009-21.
- Deveci F, Akbulut HH, Turgut T, Muz MH. Changes in serum cytokine levels in ac-

- tive tuberculosis with treatment. *Mediat Inflamm* 2005; 2005: 256-62.
- Dinareello CA, Fantuzzi G. Interleukin-18 and host defense against infection. *J Infect Dis* 2003; 187 (suppl 2): S370-84.
- Djoba Siawaya JF, Beyers N, van Helden P, Walzl G. Differential cytokine secretion and early treatment response in patients with pulmonary tuberculosis. *Clin Exp Immunol* 2009; 156: 69-77.
- Fauci AS. Host factors and the pathogenesis of HIV-induced disease. *Nature* 1996; 384: 529-34.
- Ferrero E, Biswas P, Vettoretto K, et al. Macrophages exposed to *Mycobacterium tuberculosis* release chemokines able to recruit selected leucocyte subpopulations: focus on gammadelta cells. *Immunology* 2003; 108: 365-74.
- Flynn JL, Chan J, Triebold KJ, Dalton DK, Stewart TA, Bloom BR. An essential role for interferon gamma in resistance to *Mycobacterium tuberculosis* infection. *J Exp Med* 1993; 178: 2249-54.
- Hasegawa M, Nakoshi Y, Iino T, et al. Thrombin-cleaved osteopontin in synovial fluid of subjects with rheumatoid arthritis. *J Rheumatol* 2009; 36: 240-5.
- Inomata S, Shijubo N, Kon S, et al. Circulating interleukin-18 and osteopontin are useful to evaluate disease activity in patients with tuberculosis. *Cytokine* 2005; 30: 203-11.
- Juffermans NP, Verbon A, van Deventer SJ, et al. Elevated chemokine concentrations in sera of human immunodeficiency virus (HIV)-seropositive and HIV-seronegative patients with tuberculosis: a possible role for mycobacterial lipoarabinomannan. *Infect Immun* 1999; 67: 4295-7.
- Koguchi Y, Kawakami K, Uezu K, et al. High plasma osteopontin level and its relationship with interleukin-12-mediated type 1 T helper cell response in tuberculosis. *Am J Respir Crit Care Med* 2003; 167: 1355-9.
- Lawn SD, Bekker LG, Miller RF. Immune reconstitution disease associated with mycobacterial infections in HIV-infected individuals receiving antiretrovirals. *Lancet Infect Dis* 2005; 5: 361-373.
- Li Q, Schacker T, Carlis J, Beilman G, Nguyen P, Haase AT. Functional genomic analysis of the response of HIV-1-infected lymphatic tissue to antiretroviral therapy. *J Infect Dis* 2004; 189: 572-82.
- Lin Y, Zhang M, Hofman FM, Gong J, Barnes PF. Absence of a prominent Th2 cytokine response in human tuberculosis. *Infect Immun* 1996; 64: 1351-6.
- Morosini M, Meloni F, Marone Bianco A, et al. The assessment of IFN-gamma and its regulatory cytokines in the plasma and bronchoalveolar lavage fluid of patients with active pulmonary tuberculosis. *Int J Tuberc Lung Dis* 2003; 7: 994-1000.
- Nau GJ, Chupp GL, Emile JF, et al. Osteopontin expression correlates with clinical outcome in patients with mycobacterial infection. *Am J Pathol* 2000; 157: 37-42.
- Nau GJ, Guilfoile P, Chupp GL, et al. A chemoattractant cytokine associated with granulomas in tuberculosis and silicosis. *Proc Natl Acad Sci USA* 1997; 94: 6414-9.
- Nau GJ, Liaw L, Chupp GL, Berman JS, Hogan BL, Young RA. Attenuated host resistance against *Mycobacterium bovis* BCG infection in mice lacking osteopontin. *Infect Immun* 1999; 67: 4223-30.
- O'Regan A, Berman JS. Osteopontin: a key cytokine in cell-mediated and granulomatous inflammation. *Int J Exp Pathol* 2000; 81: 373-90.
- Peresi E, Silva SM, Calvi SA, Marcondes-Machado J. Cytokines and acute phase serum proteins as markers of inflammatory regression during the treatment of pulmonary tuberculosis. *J Bras Pneumol* 2008; 34: 942-9.
- Pokkali S, Das SD. Augmented chemokine levels and chemokine receptor expression on immune cells during pulmonary tuberculosis. *Hum Immunol* 2009; 70: 110-5.
- Sahiratmadja E, Alisjahbana B, de Boer T,

- et al.* Dynamic changes in pro- and anti-inflammatory cytokine profiles and gamma interferon receptor signaling integrity correlate with tuberculosis disease activity and response to curative treatment. *Infect Immun* 2007; 75: 820-9.
- Schluger NW, Rom WN. The host immune response to tuberculosis. *Am J Respir Crit Care Med* 1998; 157: 679-91.
- Senger DR, Perruzzi CA, Papadopoulos-Sergiou A, Van de Water L. Adhesive properties of osteopontin: regulation by a naturally occurring thrombin-cleavage in close proximity to the GRGDS cell-binding domain. *Mol Biol Cell* 1994; 5: 565-74.
- Shio K, Kobayashi H, Asano T, *et al.* Thrombin-cleaved osteopontin is increased in urine of patients with rheumatoid arthritis. *J Rheumatol* 2010; 37: 704-10.
- van Crevel R, Ottenhoff TH, van der Meer JW. Innate immunity to *Mycobacterium tuberculosis*. *Clin Microbiol Rev* 2002; 15: 294-309.
- Verbon A, Juffermans N, Van Deventer SJ, Speelman P, Van Deutekom H, Van Der Poll T. Serum concentrations of cytokines in patients with active tuberculosis (TB) and after treatment. *Clin Exp Immunol* 1999; 115: 110-3.
- Weiss L, Ancuta P, Girard PM, *et al.* Restoration of normal interleukin-2 production by CD4+ T cells of human immunodeficiency virus-infected patients after 9 months of highly active antiretroviral therapy. *J Infect Dis* 1999; 180: 1057-63.
- World Health Organization (WHO). Global tuberculosis control: epidemiology, strategy, financing: WHO report 2009. Geneva: WHO, 2009.
- Yamada G, Shijubo N, Shigehara K, Okamura H, Kurimoto M, Abe S. Increased levels of circulating interleukin-18 in patients with advanced tuberculosis. *Am J Respir Crit Care Med* 2000; 161: 1786-9.