concentrations in patients with chronic TB, which had not been eradicated by treatment with conventional anti-TB drugs, and who had persistent clinical symptoms and progression of disease, were also lower than in healthy individuals. It is possible that persistence of clinical disease is associated with deficient expression of perforin and granulysin at the local site of TB infection (16). Although significant infiltration of T cells (CD3+, CD4+ and CD8+ T cells) is evident in TB lesions in patients with persistent inflammation, there are only small amounts of perforin and granulysin in these lesions, and evidence of severely impaired expression of these cytolytic effector molecules inside the distinct granules (16). Simultaneously, the numbers of granzyme A-expressing cells are increased in TB lesions, suggesting that the down-regulation of perforin and granulysin is selective and not a universal phenomenon involving all cytolytic effector molecules. These results are similar to those of recent studies which demonstrated that circulating granulysin reaches concentrations similar to those of healthy controls during TB therapy and increases further after completion of therapy (14, 15). However, larger sample sizes are necessary to gain better insight into the dynamics of plasma granulysin concentrations.

In contrast to granulysin, the concentrations of circulating IFN-γ in patients with newly diagnosed and relapsed TB were significantly higher than those of healthy controls, suggesting that IFN-γ plays a role in the regulatory and effector phases of the immune response to *Mtb* infection. In general, IFN-γ is synthesized from CD4+T cells that have been activated by recognition of mycobacterial antigen on APCs (9), as well as by CD8+ T cells from both mice and humans specific for mycobacterial antigens (17).

However, when recurrent TB was analyzed in this study, including both relapsed and chronic TB, granulysin concentrations were found to be significantly lower ($P = 0.038$, $r = -2.071$), whereas IFN-γ concentrations were significantly higher, than in controls ($P < 0.001$, $r = -4.180$, respectively), the concentrations being similar to those found in newly diagnosed TB, which is possibly due to patients with recurrent TB becoming as active as those with newly diagnosed TB. In this study, the proportional decrease in granulysin and increase in IFN-γ concentrations in newly diagnosed TB was not significantly different from that found in relapsed TB. Possible explanations are that: (i) both types of TB were active at the time of enrollment; and (ii) patients with relapsed TB had lost their immunity to *Mtb* and become active in the same way as newly diagnosed TB (because the relapsed TB patients had previous histories of newly diagnosed TB [their first episodes], re-exposure [second episode] and were registered as relapsed TB on enrollment in this study with a duration of 1–180 months [median 12 months]) between their initial treatment success and diagnosis of relapse. It is not possible to ascertain whether the episodes of relapse represented reactivation of previously inadequately treated TB, or reinfection with a new *Mtb* strain. The present results are similar to previous findings that plasma IFN-γ concentrations are significantly higher in patients with active pulmonary TB than in healthy controls and decrease after treatment. These findings might be because circulating IFN-γ comes from both local production and spill-over of IFN-γ from activated lymphocytes sequestered at the site of *Mtb* infection, as previously described (9, 14, 18). In chronic TB, circulating IFN-γ concentrations did not increase in most patients. Clearly, substantial CD4+ T cell responses occur in patients infected with *Mtb*. Failure of that response to eliminate bacteria may be partially at the level of recognition and activation of infected macrophages. *Mtb* is known to be equipped with numerous immune evasion strategies, including modulation of antigen presentation to avoid elimination by T cells. There is evidence that *Mtb*-infected macrophages have diminished ability to present antigens to CD4+ T cells, apart from IFN-γ production, which would contribute to the inability of the host to eliminate persistent infection (19).

In contrast, when PBMCs from newly diagnosed, relapsed and chronic TB were stimulated *in vitro* with PPD or H37Ra, they produced more granulysin than did stimulated controls, a finding which is in contrast to the median and individual concentrations of circulating granulysin. Possible explanations for this discrepancy are that: (i) during *in vivo* stimulation during active disease, granulysin might be rapidly consumed because of the ongoing effector immune response; (ii) *in vivo* serum granulysin is reduced during active disease because of a reduction in the T cell subset dedicated to its production (15); or (iii) when PBMCs that possibly contain primed T cells (indicated by high plasma concentrations of granulysin) are re-stimulated *in vitro* with either PPD and H37Ra, they may produce more granulysin in the supernatant. A related phenomenon has been reported in which stimulation with PPD *in vitro* PBMCs from healthy tuberculin skin test positive individuals results in increased granulysin expression in PPD-stimulated CD4+ and CD8+ T cells, compared to that of unstimulated cells (20). Moreover, it has been reported that, after stimulation *in vitro* with *Mtb* including H37Ra, both CD4+ and CD8+ T cells up-regulate mRNA expression for granulysin, granzyme A and B, perforin and CD95L (Fas ligand), and are able to lyse *Mtb* infected target cells, this being mediated primarily through the granule exocytosis pathway (21).

Median and individual concentrations of circulating IFN-γ in patients with newly diagnosed and relapsed TB were significantly higher than in healthy controls. Similar

results, namely greater IFN-γ production than in stimulated healthy controls, were seen with *in vitro* stimulation with PPD and H37Ra of PBMCs from most patients with newly diagnosed and half of relapsed TB patients, although some stimulated PBMCs from these patients produced less IFN-γ. However, the median IFN-γ production with *in vitro* stimulation of PBMCs from relapsed TB patients is lower than that of healthy controls. Surprisingly, PBMCs from healthy individuals stimulated *in vitro* with PPD and H37Ra in this study did induce significant IFN-γ production. However, these four healthy individuals were recruited from the Blood Bank of a provincial hospital in Chiang Rai where TB is endemic, and did not undergo chest X-ray, TST and any testing for latent TB infection and infection manifesting as active TB by IGRAs. At the time of recruitment, based on their histories, these individuals were thought to be healthy blood donors. However, we cannot be sure that they had never been exposed to *Mtb* and remained asymptomatic, or been vaccinated with BCG. It is known that 5–10% of those infected with *Mtb* will progress towards active TB during their lifetime, whereas the remainder are resistant to active TB, but remain infected. In fact, most Thai people are vaccinated with BCG since child. Therefore, it is possible that these healthy individuals had been exposed to *Mtb* in their lifetime, and that this had caused the high production of IFN-γ after stimulation *in vitro* with PPD and H37Ra. More normal healthy individuals from non-endemic TB areas who have been confirmed negative by chest X-ray and TST, and tested for latent TB infection and infection manifesting as active TB by IGRAs, should be included in future studies.

IFN-γ is produced from T cells (both CD4+ and CD8+T cells) and NK cells and activates bactericidal mechanisms in macrophages (3). It has been demonstrated that during the course of chronic and fatal TB infection, CD4+ T cells are absent even though CD8+ T cells can produce large amounts of IFN-γ. This supports the hypotheses that CD4+ T cells have important, non redundant roles in control of *Mtb* in addition to IFN-γ production, that CD4+ T cells assist in the development of cytotoxic CD8+ T cell populations and that the cytotoxicity exerted by effector CD8+ T cells might be an important component of anti-mycobacterial immunity (22). The present results indicate that patients with newly diagnosed and relapsed TB have low circulating granulysin but high IFN-γ concentrations before anti-TB therapy, suggesting that granulysin and IFN-γ may act in concert or in synergy in host defense against *Mtb* infection.

In conclusion, patients with active pulmonary TB have low circulating granulysin but high IFN-γ concentrations before treatment indicating their possible role in controlling *M. tuberculosis* infection.

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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,

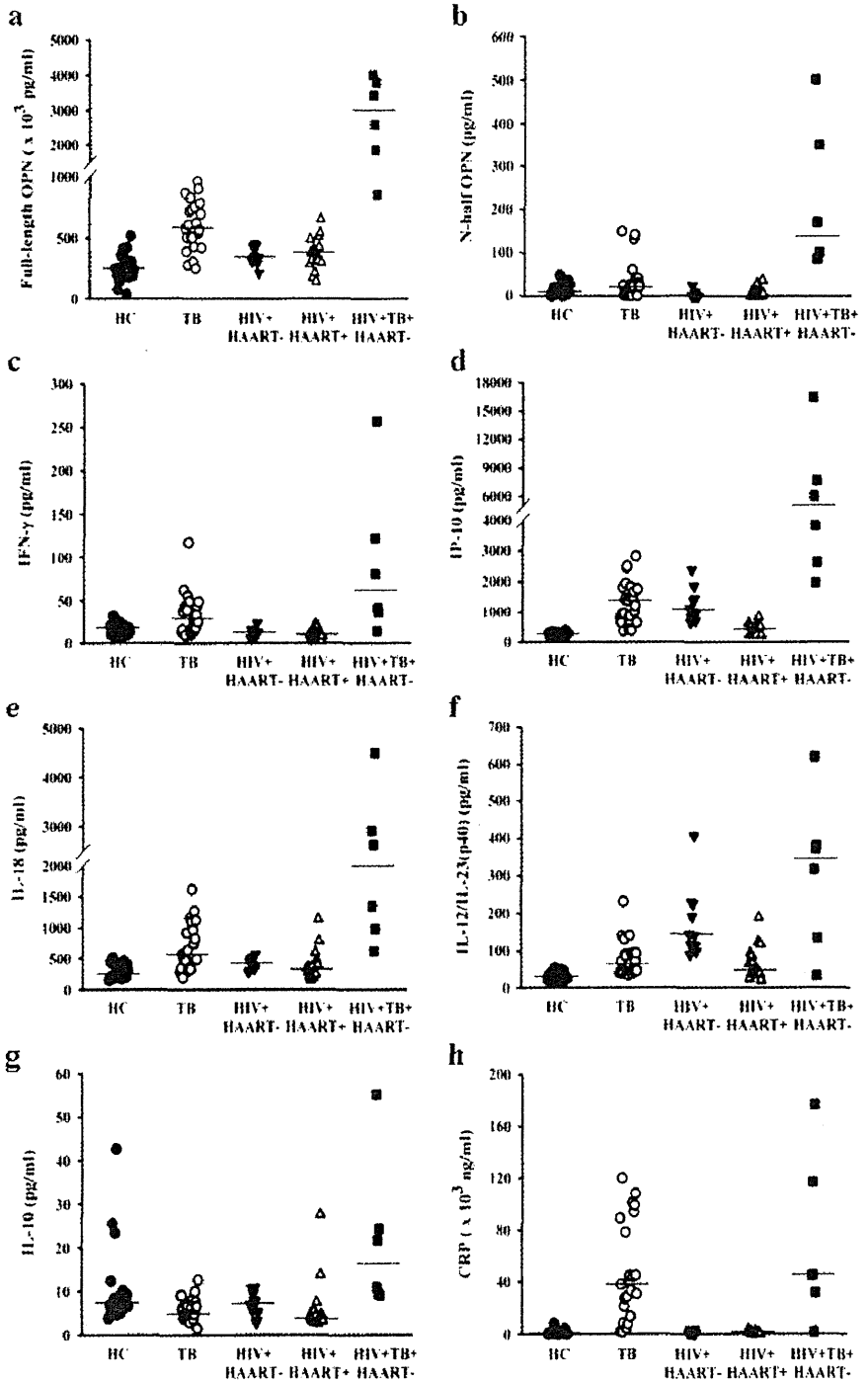

Fig 1–Circulating full-length OPN (a), N-half OPN (b), IFN-γ (c), IP-10 (d), IL-18 (e), IL-12/IL-23 (p40) (f), IL-10 (g) and CRP (h) levels in patients with tuberculosis (TB) and HIV/TB co-infection without HAART (HIV+TB+HAART-). HIV patients without HAART (HIV+HAART-) and with HAART (HIV+HAART+) were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

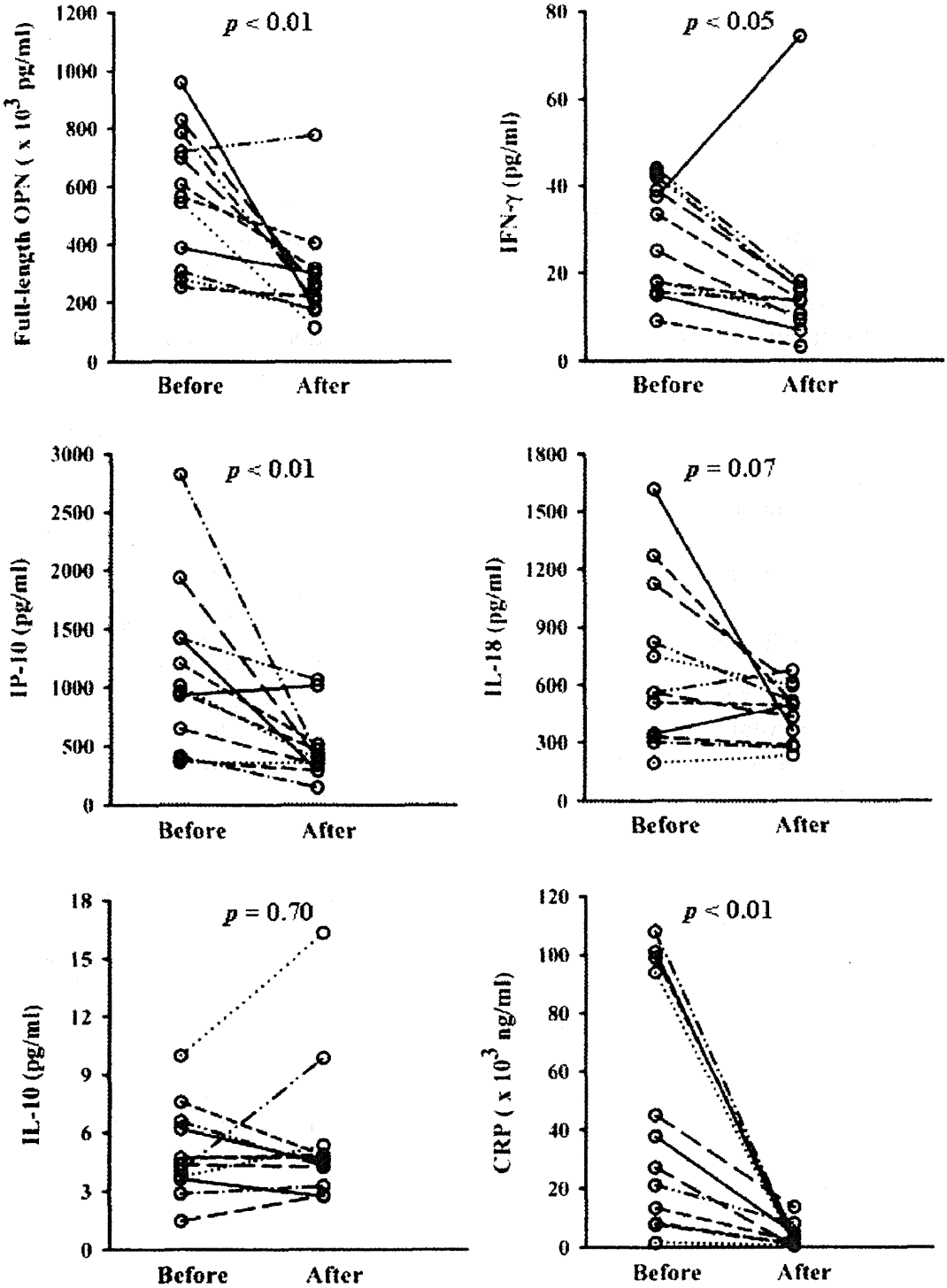

Fig 2–Circulating full-length OPN, IFN-γ, IP-10, IL-18, IL-10 and CRP levels among patients with active pulmonary TB before and after anti-TB treatment.

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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