antigen were detected with biotinylated anti-human IFN-γ monoclonal antibody and streptavidin-horseradish peroxidase conjugate (diluted to 1:250 with 10% FBS in PBS) and incubated for 1 hr at room temperature. Then, 100 μL of TMB substrate solution was added and incubated for 30 min at room temperature in the dark. The reaction was stopped by 2N H_2SO_4 solution. Samples were analyzed at 450/550 nm wavelength with a microplate ELISA reader (ELx808 IU ultra microplate reader) and IFN-γ concentrations were calculated from a standard curve using recombinant human IFN-γ. The lower detection limit was 4.7 pg/mL.

Statistical analyses

Statistical analyses were performed by SPSS software version 17.0. IFN-γ and granulysin concentrations in different independent subject groups were compared by Mann-Whitney U test. A P value < 0.05 was considered statistically significant.

RESULTS

Clinical characteristics of subjects

The clinical characteristics of the patients in the study with newly diagnosed, relapsed and chronic TB are summarized in Table 1. Infiltrates without cavitation were found on the chest radiographs of the majority of patients with newly diagnosed (57.1%) and relapsed TB (51.4%). Most patients with newly diagnosed TB (63.1%) were treated with category 1 drug regimens (2HRZE(S)/4HR) whereas relapsed (60%) and chronic TB patients (52.8%) were treated with category 2 drug regimens (2HRZES/1HRZE/5HRE). Treatment success ("cure" or "treatment completed") was achieved in 66.7%, 57.1% and 47.2% of patients with newly diagnosed, relapsed and chronic TB, respectively. Nine chronic TB patients (25.0%) had microscopically positive sputum smears at the end of their treatment course, indicating treatment failure. The median treatment duration was 7 months in patients with newly diagnosed and relapsed TB and 9 months in those with chronic TB.

Table 1. Characteristics and clinical profile of study subjects

Characteristic	Newly diagnosed TB $N = 84$	Relapsed TB $N = 35$	Chronic TB $N = 36$
Sex			
Male	60	27	15
Female	24	8	21
Age (years)			
Median	44	48	49
Range	9–85	28–88	14–82
Chest X-ray findings			
Infiltrate/Non-cavitating	48	18	15
Cavitating	14	8	6
Not documented	22	9	15
Treatment regimens			
2HRZE(S)/4HR[a] (CAT1)	53	10	
2HRZES/1HRZE/5HRE[a] (CAT2)	19	21	19
2HRZ/2HR[a] (CAT3)			
Second line drug (CAT4)	12	4	17
Duration of treatment (months)			
Median	7	7	9
Range	0–26	0–14	5–20
Treatment outcomes			
Cure	51	18	14
Completed	5	2	3
Default	10	5	7
Died	4	6	3
Failure	7	3	9
Not documented	7	1	

[a]The standard code for TB treatment regimens, each anti-TB drug has an abbreviation: streptomycin (S), isoniazid (H), rifampicin (R), pyrazinamide (Z) and ethambutol (E). CAT, category.

Circulating granulysin concentrations in clinical tuberculosis before anti-tuberculosis therapy

The concentrations of circulating granulysin in patients with newly diagnosed TB (median ± SE = 1.511 ± 0.287 ng/mL, range 0.560–15.600 ng/mL) and relapsed TB (median ± SE = 1.458 ± 0.329 ng/mL, range 0.403–8.110 ng/mL) were significantly lower than those of healthy controls (median ± SE = 2.470 ± 0.186 ng/mL, range 0.662–5.055 ng/mL) ($P < 0.001$, $r = -3.816$ and $P = 0.004$, $r = -2.853$, respectively). Patients with chronic TB (median ± SE = 1.917 ± 0.264 ng/mL, range 0.549–6.970 ng/mL) had lower granulysin concentrations than controls, this difference not being significant ($P = 0.442$, $r = -0.769$). Median concentrations of granulysin were similar in patients with newly diagnosed and relapsed TB, but both were significantly lower than in chronic TB ($P = 0.003$, $r = -2.967$ and $P = 0.022$, $r = -2.294$, respectively) (Fig. 1).

Granulysin production in peripheral blood mononuclear cell stimulation assay

Granulysin production in PBMCs stimulated *in vitro* with PPD and H37Ra were measured in 46 patients with newly diagnosed, 21 with relapsed and 8 with chronic TB. Granulysin production by newly diagnosed TB-PBMCs stimulated *in vitro* with PPD (median ± SE = 0.796 ± 0.071 ng/mL, range 0.208–2.196 ng/mL) and H37Ra (median ± SE = 0.976 ± 0.065 ng/mL, range 0.246–1.823 ng/ml) were significantly higher than those of

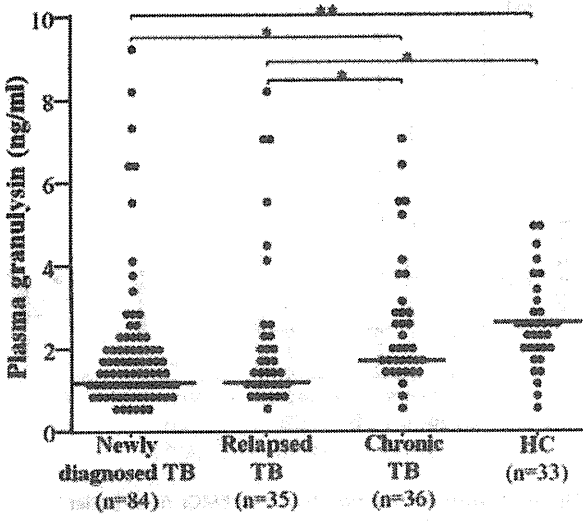

Fig. 1. Circulating granulysin concentrations in patients with newly diagnosed, relapsed and chronic TB in comparison with healthy controls. Each dot represented one individual. The horizontal bars indicate the median of each group. *, $P < 0.05$; **, $P < 0.001$; HC, healthy control.

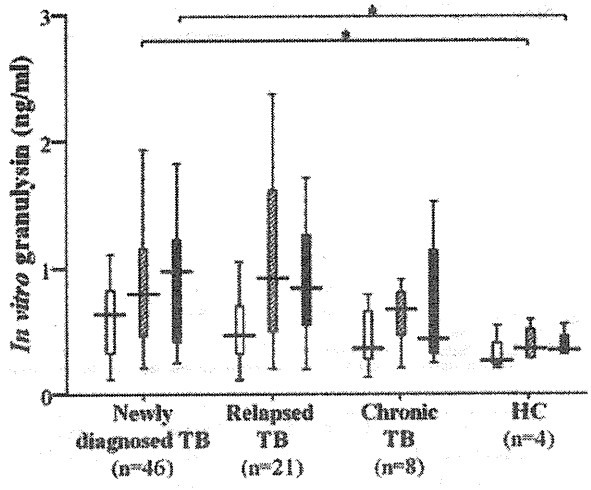

Fig. 2. *In vitro* granulysin production by PBMCs from patients with newly diagnosed, relapsed and chronic TB and healthy individuals stimulated with PPD (diagonal shading) and heat killed *Mycobacterium tuberculosis* (H37Ra) (black). Supernatant from PBMCs without stimulation was used as controls (clear). The horizontal bars indicate the median of each group. *, $P < 0.05$.

healthy controls stimulated *in vitro* with PPD (median ± SE = 0.359 ± 0.073 ng/mL, range 0.283–0.591 ng/mL), and H37Ra (median ± SE = 0.348 ± 0.056 ng/mL, range 0.320–0.559 ng/mL) ($P = 0.022$, $r = -2.289$ and $P = 0.032$, $r = -2.146$, respectively). Controls were PBMC supernatants from healthy controls without stimulation (median ± SE = 0.262 ± 0.076 ng/mL, range 0.206–0.542 ng/mL) and PBMC supernatants from newly diagnosed TB patients without stimulation (median ± SE = 0.636 ± 0.051 ng/mL, ranged 0.117–1.665 ng/mL). Although granulysin production by relapsed TB-PBMCs stimulated *in vitro* with PPD (median ± SE = 0.922 ± 0.146 ng/mL, range 0.205–2.374 ng/mL) and H37Ra (median ± SE = 0.841 ± 0.123 ng/mL, range 0.197–2.324 ng/mL) were higher than those of healthy controls, these differences were not significant ($P = 0.054$, $r = -1.927$ and $P = 0.081$, $r = -1.742$, respectively). PBMCs of patients with chronic TB stimulated *in vitro* with PPD (median ± SE = 0.674 ± 0.120 ng/mL, range 0.475–1.345 ng/mL) and H37Ra (median ± SE = 0.435 ± 0.173 ng/mL, range 0.408–1.521 ng/mL) produced greater amounts of granulysin than did healthy controls, the difference not being significant ($P = 0.089$, $r = -1.698$ and $P = 0.497$, $r = -0.679$, respectively). Similar median amounts of granulysin were produced by PBMCs of newly diagnosed and relapsed TB stimulated *in vitro* with PPD and H37Ra but higher amounts by PBMCs of chronic TB, the difference not being significant (newly diagnosed and chronic TB: $P = 0.330$, $r = -0.974$ for PPD and $P = 0.242$, $r = -1.169$ for H37Ra; relapsed and chronic TB: $P = 0.232$, $r = -1.196$ for PPD and $P = 0.380$, $r = -0.878$ for H37Ra) (Fig. 2).

Circulating interferon-γ concentrations in clinical tuberculosis before anti-TB therapy

In contrast to granulysin, the circulating IFN-γ concentrations in patients with newly diagnosed TB (median ± SE = 6.15 ± 4.58 pg/mL, range < 4.7–300 pg/mL) and relapsed TB (median ± SE = 7.93 ± 8.86 pg/mL, range <4.7–310.73 pg/mL) were significantly higher than those of healthy controls (median ± SE = <4.7 ± 0.20 pg/mL, range <4.7–10.13 pg/mL) ($P < 0.001$, $r = -3.923$ and $P < 0.001$, $r = -4.325$, respectively). Circulating IFN-γ concentrations in most chronic TB patients were similar to those of healthy individuals (median ± SE = <4.7 ± 3.76 pg/mL, range <4.7–123.69 pg/mL) ($P = 0.051$, $r = -3.486$). The median concentrations of IFN-γ were similar in patients with newly diagnosed and relapsed TB, but both were higher than in chronic TB, the difference not being significant ($P = 0.395$, $r = -0.851$ and $P = 0.333$, $r = -0.968$, respectively) (Fig. 3).

Interferon-γ production in peripheral blood mononuclear cell stimulation assay

The median IFN-γ production by PBMCs of newly diagnosed TB patients stimulated *in vitro* with PPD (median ± SE = 535 ± 94 pg/mL, range <4.7–2400 pg/mL) was higher than that of healthy controls (median ± SE = 434 ±

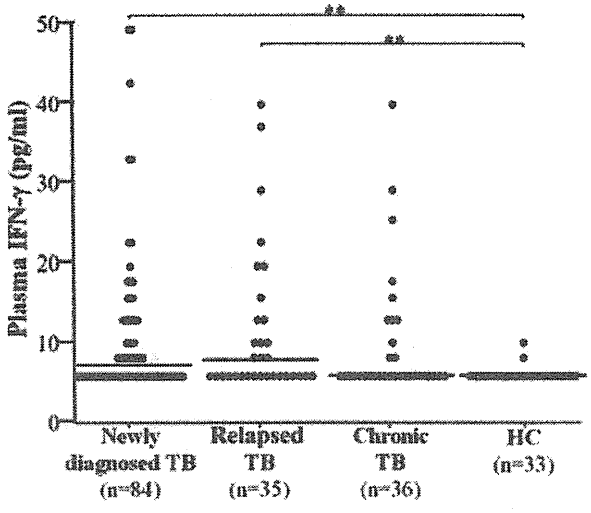

Fig. 3. Circulating IFN-γ concentrations in patients with newly diagnosed, relapsed and chronic TB in comparison with healthy controls. Each dot represents one individual. The horizontal bars indicate the median of each group. **, $P < 0.001$; HC, healthy control.

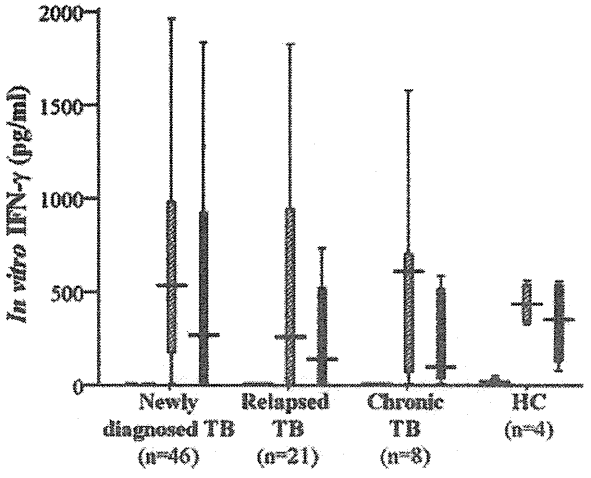

Fig. 4. *In vitro* IFN-γ production by PBMCs from patients with newly diagnosed, relapsed and chronic TB and healthy individuals stimulated with PPD (diagonal shading) and H37Ra (black). Supernatant from PBMCs without stimulation was used as controls (clear). The horizontal bars indicate the median of each group.

57 pg/mL, range 326–562 pg/mL) ($P = 0.591$, $r = -0.537$). However, most newly diagnosed TB-PBMCs stimulated *in vitro* with H37Ra produced higher IFN-γ concentrations (range <4.7–8025 pg/mL), but the median was similar (median ± SE = 270 ± 260 pg/mL) to that of healthy controls (median ± SE = 351 ± 120 pg/mL, range 76–556 pg/mL) ($P = 0.914$, $r = -0.107$). Supernatant from PBMCs without stimulation was used as a cell control (median ± SE = 14.29 ± 8.88 pg/mL, range 9.85–48.06 pg/mL), while supernatant from newly diagnosed TB-PBMCs without stimulation was used as a control for IFN-γ production (median ± SE = <4.7 ± 5.08 pg/mL, range <4.7–231 pg/mL). IFN-γ production by PBMCs from half the patients with relapsed TB stimulated either with PPD (range <4.7–4225 pg/mL) or H37Ra (range <4.7–2575 pg/mL) was higher than that of normal controls. However, their medians (median ± SE = 260 ± 258 pg/mL for PPD, and median ± SE = 138 ± 136 pg/mL for H37Ra) were lower than those of healthy controls; these differences were not significant ($P = 0.823$, $r = -0.223$ and $P = 0.412$, $r = -0.821$, respectively). Chronic TB-PBMCs stimulated *in vitro* with PPD (median ± SE = 610 ± 166 pg/mL, range <4.7–1575 pg/mL) produced higher IFN-γ concentrations than did healthy controls, and some PBMCs stimulated *in vitro* with H37Ra also produced higher IFN-γ concentrations (range <4.7–1835 pg/mL) although the median was lower (median ± SE = 95 ± 198 pg/mL) than that of healthy controls ($P = 0.758$, $r = -0.309$ and $P = 0.354$, $r = -0.927$, respectively). Similar median amounts of IFN-γ production by PBMCs of newly diagnosed and chronic TB stimulated *in vitro* with PPD were found, and these were higher than for relapsed TB, the difference not being significant ($P = 0.436$, $r = -0.779$ and $P = 0.928$, $r = -0.091$, respectively). The median amount of IFN-γ produced by PBMCs of newly diagnosed TB stimulated *in vitro* with H37Ra was higher than that for relapsed and chronic TB ($P = 0.202$, $r = -1.275$ and $P = 0.982$, $r = -0.023$, respectively) (Fig. 4).

DISCUSSION

In this study, the correlations of plasma granulysin and IFN-γ concentrations with clinical disease in patients with newly diagnosed pulmonary, relapsed and chronic TB in northern Thailand, where TB is endemic, were evaluated. The effects of *in vitro* stimulation with PPD and H37Ra of PBMCs from these patients were also investigated. The finding of decreased circulating granulysin and increased IFN-γ in patients with newly diagnosed, relapsed and chronic TB before anti-TB therapy indicated involvement of granulysin and IFN-γ in host defense against TB infections.

In patients with newly diagnosed and relapsed pulmonary TB who had not yet received anti-TB therapy, plasma granulysin concentrations were significantly decreased compared to those of healthy individuals. This may be because granulysin is rapidly consumed during active disease, because of an ongoing effector immune response, or because plasma granulysin is reduced during active disease because of a reduction in the T cell subset dedicated to its production (15). However, granulysin

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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Review Article

Innate Immune Effectors in Mycobacterial Infection

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Tuberculosis, which is caused by infection with *Mycobacterium tuberculosis* (Mtb), remains one of the major bacterial infections worldwide. Host defense against Mtb is mediated by a combination of innate and adaptive immune responses. In the last 15 years, the mechanisms for activation of innate immunity have been elucidated. Toll-like receptors (TLRs) have been revealed to be critical for the recognition of pathogenic microorganisms including mycobacteria. Subsequent studies further revealed that NOD-like receptors and C-type lectin receptors are responsible for the TLR-independent recognition of mycobacteria. Several molecules, such as active vitamin D_3, secretary leukocyte protease inhibitor, and lipocalin 2, all of which are induced by TLR stimulation, have been shown to direct innate immune responses to mycobacteria. In addition, Irgm1-dependent autophagy has recently been demonstrated to eliminate intracellular mycobacteria. Thus, our understanding of the mechanisms for the innate immune response to mycobacteria is developing.

1. Introduction

In humans, tuberculosis is one of deadly infectious diseases. Indeed, approximately 2 million tuberculosis patients die every year. The risk of disease is also increased by emergence of acquired immune deficiency syndrome and development of multidrug-resistant mycobacteria [1]. Therefore, it is important to understand the host defense mechanisms against mycobacteria. Inhalation of aerosols containing *Mycobacterium tuberculosis* (Mtb) causes tuberculosis. After inhalation, Mtb invades alveolar macrophages to enter into the host and establish the infection. The host, in turn, ignites defense responses through sequential activation of immunity, a combination of innate and adaptive immune systems. In the adaptive phase of immune responses, the importance of Th1/IFN-γ-mediated responses in mycobacterial infection has been well established [2]. In contrast, although macrophages are the major target of invasion by Mtb, how the innate arm of immunity mediates host defense against mycobacteria had long remained unknown. However, the mechanisms behind innate immune responses have been revealed in the past 15 years following the identification and characterization of pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) [3]. Furthermore, it has been elucidated that TLR-dependent activation of innate immunity controls the development of adaptive immune responses [4]. The involvement of PRRs other than TLRs in the recognition of mycobacteria has also been revealed. In addition to the induction of adaptive immune responses, the PRR recognition of mycobacteria induces expression of several effector molecules participating in the innate host responses. The role of these innate effector molecules in mycobacterial infection is being elucidated. PRR-independent mechanisms for mycobacterial killing, such as autophagy, have also been revealed. In this paper, we will describe recent advances in our understanding of effectors that mediate innate immune responses against mycobacteria.

2. Toll-Like Receptors in Mycobacterial Infection

Innate immune responses after mycobacterial infection are initiated by recognition of mycobacterial components by PRRs, with mycobacterial components activating several

TLRs (Figure 1). Genomic DNA from a *Mycobacterium bovis* strain, bacillus Calmette–Guérin (BCG), have an ability to augment NK cell activity and induce type I IFNs from murine spleen cells and human peripheral blood lymphocytes. The immunostimulatory activity of mycobacterial DNA was ascribed to the presence of palindromic sequences including the 5′-CG-3′ motif, now called CpG motif [5], and now known to activate TLR9 [6]. The mycobacterial cell wall consists of several glycolipids. Among these, lipoarabinomannan (LAM) lacking mannose end capping, lipomannan (LM), and phosphatidyl-*myo*-inositol mannoside (PIM) are recognized by TLR2 [7, 8]. The 19-kDa lipoprotein of Mtb also activates macrophages via TLR2 [9, 10]. TLR4 is also presumed to recognize mycobacterial components.

The *in vivo* importance of the TLR-mediated signal in host defense to Mtb was highlighted in studies using mice lacking MyD88, a critical component of TLR signaling. MyD88-deficient mice are highly susceptible to airborne infection with Mtb [11–13]. In contrast to mice lacking MyD88, mice lacking individual TLRs are not dramatically susceptible to Mtb infection. Susceptibility of TLR2-deficient mice to Mtb infection varies between different studies [14, 15], while TLR4-deficient mice do not show high susceptibility to Mtb infection [16, 17]. A report demonstrates that TLR9-deficient mice are susceptible to Mtb infection and mice lacking both TLR2 and TLR9 are more susceptible [18]. These findings indicate that multiple TLRs might be involved in mycobacterial recognition. However, a recent report using mice lacking TLR2/TLR4/TLR9 indicated that these triple KO mice show a milder phenotype than MyD88-deficient mice [12]. Therefore, more intensive examination is required to reveal whether TLRs or molecules other than TLRs activating MyD88 mediate innate immune responses to mycobacterial infection. This study also demonstrated that Th1-like adaptive immune responses are induced even in Mtb-infected MyD88-deficient mice [12]. Therefore, the TLR/MyD88-independent component of innate immunity is involved in the induction of adaptive immune responses during mycobacterial infection. The TLR/MyD88-independent response might be induced by other PRRs described below.

3. Non-TLRs in Mycobacterial Infection

Several recent findings have indicated that PRRs other than TLRs evoke innate immune responses [19]. These include RIG-I-like receptors, NOD-like receptors (NLRs), and C-type lectin receptors. Among these PRRs, NOD-like receptors and C-type lectin receptors have been implicated in the innate recognition of mycobacteria (Figure 2).

NOD2 is a member of NLRs that recognize muramyl dipeptide (MDP), a core component of bacterial peptidoglycan, in the cytoplasmic compartment. Macrophages from NOD2-deficient mice show a defective cytokine production after Mtb infection [20]. Similarly, mononuclear cells of individuals homozygous for the *3020insC NOD2* mutation show a defective cytokine response after stimulation with Mtb [7]. Activation of the NOD2-mediated pathway is induced by stimulation with live Mtb, but not by heat-killed Mtb [8]. Live Mtb, which is localized in the phagosomal compartment within macrophages, stimulates the cytosolic NOD2 pathway by inducing phagosomal membrane damage [21]. The NOD2 ligand MDP is N-acetylated in most bacteria. However, MDP is N-glycolylated by N-acetyl muramic acid hydroxylase (NamH) in mycobacteria. Analyses using *M. smegmatis* namH mutant and NOD2-deficient mice showed that N-glycolyl MDP is recognized by NOD2. In addition, N-glycolyl MDP is the more potent NOD2 activator than N-acetyl MDP [22]. Thus, NOD2 contributed to the recognition of mycobacteria.

Several members of the NLR family, such as NLRP1, NLRP3, and IPAF, induce assembly of the inflammasome, which leads to caspase-1-dependent secretion of IL-1β and IL-18 [23]. The involvement of IL-1β and IL-18 in mycobacterial infection was demonstrated in studies using knockout mice [24–27]. A recent study demonstrated that mycobacteria inhibit the inflammasome-dependent casapase-1 activation leading to defective IL-1β production [28]. The inhibition of caspase-1 activation has further been shown to be mediated by an Mtb gene, *zmp1*, which encodes a putative Zn^{2+} metalloprotease. Thus, Mtb has a strategy that evades the inflammasome-mediated innate immune responses.

C-type lectin receptors, such as mannose receptor, were originally reported to mediate phagocytosis of mycobacteria [29]. Another C-type lectin receptor, DC-SIGN, has been shown to recognize mycobacteria, and thereby modulate the function of dendritic cells [30–32]. Recognition of mycobacteria by dectin-1 has been shown to induce gene expression such as TNF-α, IL-6, and IL-12 [33, 34]. In addition, macrophage inducible C-type lectin (Mincle) has recently been shown to recognize trehalose-6,6′-dimycolate (TDM: also called cord factor), a mycobacterial cell wall glycolipid that is the most studied immunostimulatory component of Mtb [35, 36], thereafter modulating macrophage activation. Thus, several C-type lectin receptors are involved in the recognition of mycobacteria.

CARD9 is involved in the signaling pathways of several PRRs including TLRs, NOD-like receptors, and FcRγ-associated C-type lectin receptors through association with Bcl-10 and MALT. Therefore, it is not surprising that CARD9-deficient mice are highly susceptible to Mtb infection. However, interestingly the high susceptibility of CARD9-deficient mice to the infection has been shown to be excessive inflammatory responses due to defective production of the immunosuppressive cytokine IL-10 [37]. Mincle is a member of C-type lectin receptors associated with FcRγ [38]. Accordingly, TDM-induced immune responses are mediated by the signaling pathway activating CARD9 [36, 39].

TLRs and C-type lectin receptors are expressed on the plasma membrane or the endosomal/phagosomal membrane, whereas NOD-like receptors are expressed within the cytoplasm. Indeed, distinct patterns of TLR- and NOD-like receptor-mediated gene expression profiles have been demonstrated in infection with intracellular bacteria [40]. Thus, several PRRs recognize mycobacteria in distinct sites within the host cells (macrophages) to synergistically induce effective host defense responses.

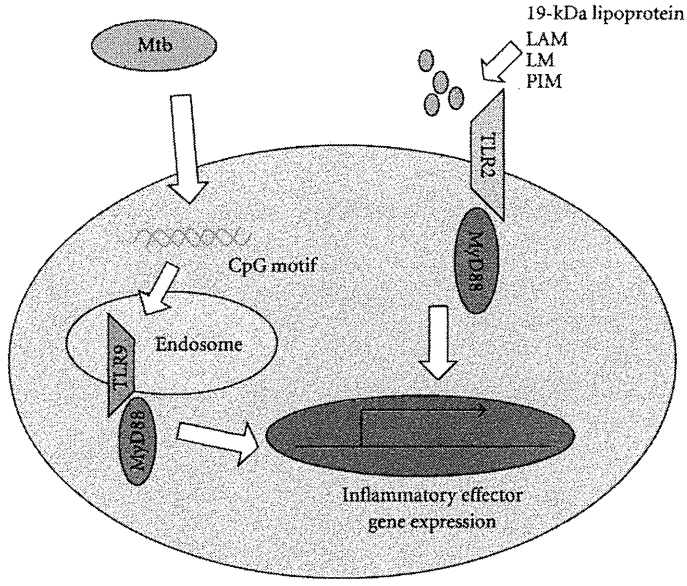

FIGURE 1: Recognition of mycobacteria by Toll-like receptors. TLR2 recognizes several mycobacterial-derived components. TLR9 recognizes mycobacterial DNA including the CpG motif within endosomal compartments. TLR-dependent recognition of mycobacteria induces activation of signaling pathways via the adaptor molecule MyD88, leading to activation of gene expression.

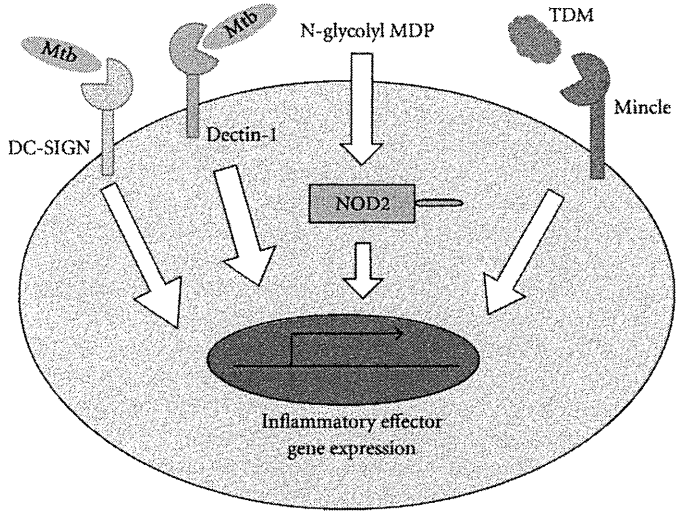

FIGURE 2: Recognition of mycobacteria by pattern recognition receptors. Several pattern recognition receptors, such as NOD-like receptors and C-type lectin receptors, mediate the TLR-independent recognition of mycobacteria. NOD2, a member of NOD-like receptors, recognizes mycobacterial N-glycolyl MDP within the cytoplasm. DC-SIGN and dectin-1 are members of C-type lectin receptors, which are implicated in the recognition of mycobacteria. In addition, Mincle has been shown to recognize TDM (a mycobacterial cell wall glycolipid).

4. Effectors for Mycobacterial Killing

The recognition of mycobacteria by several PRRs induces the expression of several genes that mediate host defense (Figure 3). Among these gene products, vitamin D receptor (VDR) and Cyp27b1, a 25-hydroxyvitamin D_3 1-α-hydroxylase that catalyzes inactive provitamin D into the bioactive form of vitamin D (1, 25 $(OH)_2D_3$), have been shown to be induced by TLR2 ligands in human macrophages [41]. Stimulation of macrophages with 1, 25 $(OH)_2D_3$ induces the expression of the antimicrobial peptide cathelicidin, and thereby enhances the antimycobacterial killing activity [42]. In addition to cathelicidin, the small cationic antimicrobial peptide defensin mediates innate immune responses to Mtb [43, 44]. Experimental infection of the lung epithelial cell line A549 with Mtb strongly induces production of human β-defensin HBD-2, which leads to Mtb killing [43]. HBD-2 expression has also been shown to be induced by TLR2 [45].

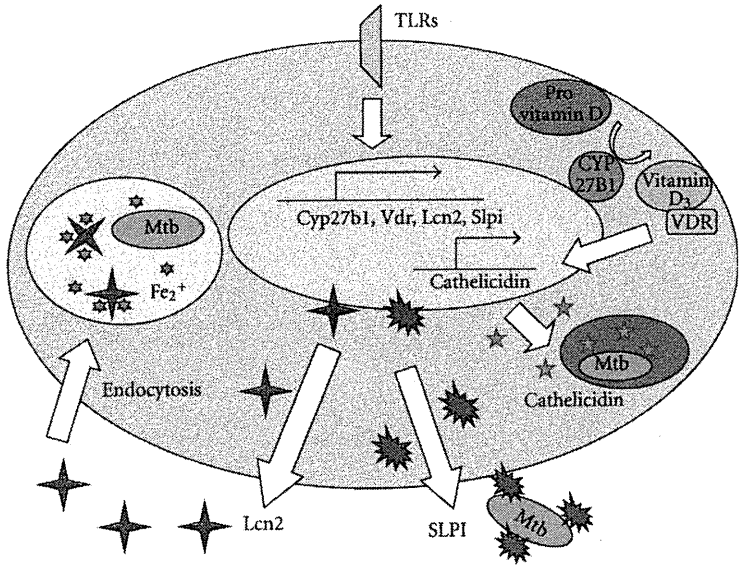

FIGURE 3: TLR-dependent innate response to mycobacteria. Several TLR-dependent gene products mediate innate immune responses to mycobacteria. Mycobacterial stimulation of TLR2 induces expression of Cyp27b1 and vitamin D receptor (VDR), both of which are involved in vitamin D_3-dependent induction of cathelicidin which directly kills mycobacteria. TLR-dependent induction of SLPI mediates disruption of the mycobacterial cell wall. Lcn2, which is also induced by TLR stimulation, is internalized into the alveolar epithelial cells and inhibits mycobacterial growth by sequestering iron uptake.

Gene expression analyses of the lung of mycobacteria-infected mice have identified several TLR-dependent genes that are involved in innate immune responses during mycobacterial infection. These genes include *Slpi*, encoding secretory leukocyte protease inhibitor (SLPI), and *Lcn2*, encoding lipocalin 2 (Lcn2). SLPI is a secreted protein composed of two cysteine-rich whey acidic protein (WAP) domains [46–48]. SLPI was named after its presence in secretions and its function as a serine protease inhibitor. SLPI was originally shown to mediate wound healing [49, 50]. SLPI is produced by bronchial and alveolar epithelial cells as well as alveolar macrophages and is secreted into the alveolar space at the early phase of mycobacterial respiratory infections. Recombinant mouse SLPI effectively inhibits the *in vitro* growth of BCG and Mtb through disruption of the mycobacterial cell wall structure. Cationic residues within the WAP domains of SLPI are essential for the disruption of mycobacterial cell walls. Moreover, SLPI-deficient mice are highly susceptible to mycobacterial infection [51]. The mechanism by which SLPI attaches to the membrane of mycobacteria has been elucidated. SLPI recognizes mannan-capped lipoarabinomannans and phosphatidylinositol mannoside, which are conserved in mycobacteria. Thus, SLPI might act as a PRR in order to bind to the mycobacterial membrane [52].

Lcn2 (also known as neutrophil gelatinase-associated lipocalin, 24p3, or siderocalin) was originally identified in the granules of human neutrophils. Lcn2 is a member of the lipocalin protein family and able to bind to small hydrophobic molecules, siderophore. It is a bacterial molecule made in iron-limited environment and facilitates iron uptake by bacteria [53–58]. The expression of Lcn2 is increased in macrophages of LPS-treated mice [59]. In addition, it is secreted into the alveolar space by alveolar macrophages and epithelial cells during the early phase of respiratory mycobacterial infection. Lcn2 inhibits *in vitro* growth of Mtb by binding the mycobacterial siderophore carboxymycobactin, thereby sequestering iron uptake. Moreover, Lcn2-deficient mice are highly susceptible to intratracheal infection with Mtb. Lcn2 is internalized into alveolar epithelial cells by endocytosis and colocalized with mycobacteria within the cells. Therefore, Lcn2 presumably sequesters iron uptake of mycobacteria within epithelial cells and thereby inhibits their intracellular growth. Within macrophages, the endocytosed Lcn2 and mycobacteria show distinct patterns of subcellular localization, which might allow growth of mycobacteria within macrophages [60]. Thus, Lcn2, which is secreted into the alveolar space during the early phase of mycobacterial infection, is endocytosed into alveolar epithelial cells, thereby inhibiting mycobacterial growth [61].

5. Autophagy in Mycobacterial Infection

Phagocytosis of myobacteria and PRR-dependent recognition of mycobacteria activate several effector functions in macrophages (Figure 4). Maturation of phagosomes is a crucial step in the elimination of intracellular bacteria. The natural-resistance-associated macrophage protein (Nramp1), which is encoded by Slc11a1, is thought to mediate transportation of divalent cations in the phagosomal membrane and thereby sequesters iron (Fe^{2+}) from mycobacteria to enhance bacterial killing by macrophages [62]. Polymorphisms of the *SLC11A1* gene have been associated with susceptibility to several infectious diseases,

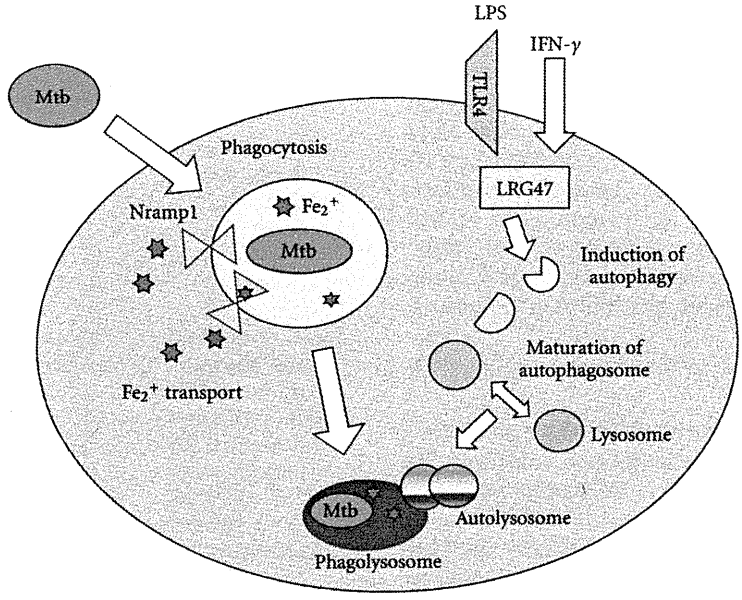

FIGURE 4: Effectors that mediate mycobacterial killing in macrophages. Macrophages eliminate invading mycobacteria by activating several effector functions, such as phagosomes and autophagy. Nramp1 is expressed in the phagosomal membrane and presumably mediates mycobacterial killing by sequestering iron uptake. IFN-γ and the TLR4 ligand induce expression of LRG47, which in turn stimulates autophagy in macrophages. Autophagy is responsible for mycobacterial killing by promoting fusion of mycobacterial phagosomes to lysosomes.

including tuberculosis [63, 64]. However, *in vivo* studies have shown that Nramp1-deficient mice are not more susceptible than wild-type mice to infection with virulent Mtb [65]. Thus, the role of Nramp1 in mycobacterial infection is still controversial. This might be due to the presence of other killing mechanisms for mycobacteria in macrophages. Indeed, autophagy has recently been shown to be involved in host defense against several intracellular pathogens that reside within phagosomes [66]. Autophagy was originally identified as a homeostatic mechanism for the catabolic reaction of cellular constitutes [67, 68]. It has been demonstrated that autophagy mediates innate immune responses against mycobacteria by promoting phagolysosomal maturation within macrophages [69, 70]. Autophagy is induced by IFN-γ-dependent induction of a member of the immunity-related p47 guanosine triphosphatases (IRG) family, LRG47 (also known as Irgm1) in murine macrophages [69]. The importance of LRG47 in resistance to Mtb infection was demonstrated in LRG47-deficient mice, which show high susceptibility to infection [71]. A subsequent study demonstrated that stimulation of macrophages with the TLR4 ligand LPS leads to the MyD88-independent induction of autophagy, which enhances mycobacterial colocalization with the autophagosomes. Since LPS stimulation induces expression of LRG47, the TLR signaling establishes a close relationship between innate immunity and autophagy in mycobacterial infection [72]. In humans, the most equivalent gene to murine Irgm1 is IRGM. IRGM has also been implicated in the induction of autophagy in mycobacteria-infected human macrophages [73]. Irgm1 has been shown to associate with the mycobacterial phagosome by interacting with phosphatidylinositol-3,4-bisphosphate (PtdIns(3,4)P(2)) and PtdIns(3,4,5)P(3) [74]. The connection of the IRG family of proteins with autophagy has been further demonstrated in an alternative intracellular infection model. In this study, Irgm3 (also known as IGTP) has been implicated in autophagy induction in macrophages infected with *Toxoplasma gondii* [75].

p62 (also called A170 or SQSTM1) directly binds to cytosolic polyubiquitinated proteins and thereby induces their autophagic clearance [76, 77]. It has also been shown that p62 targets intracellular *Salmonella typhimurium* decorated by ubiquitinated proteins to induce autophagy [78]. In the case of mycobacteria residing in the phagosome, p62 delivers cytosolic ubiquitinated proteins to autophagolysosomes where they are proteolytically processed to products that are able to kill mycobacteria [79]. In accordance with this finding, it has been shown that mycobacterial killing by ubiquitin-derived peptides is enhanced by autophagy [80].

As described above, 1, 25 $(OH)_2D_3$ mediates antimycobacterial activity via induction of cathelicidin. A recent report demonstrated that 1, 25 $(OH)_2D_3$-mediated expression of cathelicidin induces autophagy [81]. Thus, several innate immune effectors are closely interacted.

6. Human Genetics in Tuberculosis

In addition to the intensive studies using murine models, considerable advances have been made in our understanding of the susceptibility to Mtb infection in humans through the identification of mutations and polymorphisms of

innate immunity-related genes in tuberculosis patients. As described above, polymorphisms of the *SLC11A1* gene are associated with tuberculosis. Subsequent studies identified a significant distinction between tuberculosis patients and healthy controls in *TLR2* Arg753Gln polymorphism genotype, indicating that the *TLR2* polymorphism influences the susceptibility of Mtb infection [82]. *VDR* polymorphisms have also been implicated in the susceptibility of Mtb infection [83]. These studies suggest that several genes, which have been revealed to be critical in innate responses in mouse models of Mtb infection, regulate Mtb infection in humans.

7. Conclusion

Since the discovery of TLRs at the end of the 20th century, rapid advances have been made in our understanding of the mechanisms for activation of innate immunity. Accordingly, innate immunity has been revealed to have a pivotal role in host defense against mycobacteria. The TLR-independent mechanisms for the innate immune response to mycobacteria have also been elucidated. The emergence of multidrug-resistant Mtb is now a major public health problem all over the world. In this context, it is highly critical to develop a new strategy for the treatment of Mtb-infected patients that supplements the conventional antimycobacterial chemotherapeutic drugs. More precise understanding of the innate immune response to Mtb will pave the way for the development of an effective drug that targets the host innate immunity for the treatment of tuberculosis.

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A Histone-Like Protein of Mycobacteria Possesses Ferritin Superfamily Protein-Like Activity and Protects against DNA Damage by Fenton Reaction

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Abstract

Iron is an essential metal for living organisms but its level must be strictly controlled in cells, because ferrous ion induces toxicity by generating highly active reactive oxygen, hydroxyl radicals, through the Fenton reaction. In addition, ferric ion shows low solubility under physiological conditions. To overcome these obstacles living organisms possess Ferritin superfamily proteins that are distributed in all three domains of life: bacteria, archaea, and eukaryotes. These proteins minimize hydroxyl radical formation by ferroxidase activity that converts Fe^{2+} into Fe^{3+} and sequesters iron by storing it as a mineral inside a protein cage. In this study, we discovered that mycobacterial DNA-binding protein 1 (MDP1), a histone-like protein, has similar activity to ferritin superfamily proteins. MDP1 prevented the Fenton reaction and protects DNA by the ferroxidase activity. The K_m values of the ferroxidase activity by MDP1 of *Mycobacterium bovis* bacillus Calmette-Guérin (BCG-3007c), *Mycobacterium tuberculosis* (Rv2986c), and *Mycobacterium leprae* (ML1683; ML-LBP) were 0.292, 0.252, and 0.129 mM, respectively. Furthermore, one MDP1 molecule directly captured 81.4±19.1 iron atoms, suggesting the role of this protein in iron storage. This study describes for the first time a ferroxidase-iron storage protein outside of the ferritin superfamily proteins and the protective role of this bacterial protein from DNA damage.

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Introduction

Iron is an essential element for virtually all living organisms, and is convertible between the ferrous ion (Fe^{2+}) and ferric ion (Fe^{3+}) under physiological pH. Living organisms employ this feature of iron for the catalytic centers of several critical enzymes, such as ribonucleotide reductase for the synthesis of DNA substrates and cytochromes involved in electron transport in respiration.

However, iron levels must be strictly regulated as it is potentially toxic to cells. Fe^{3+} is stable but forms insoluble hydroxy-aquo complexes and its solubility is under 10^{-18} M in physiological conditions; bacterial multiplication requires approximately 10^{-7} M iron [1]. Fe^{2+} is a reductant and generates reactive oxygen species (ROS) and reactive nitrogen species (RNS), which damage most cell components, including DNA, membranes, and proteins. In particular, the hydroxyl radical (•OH) is known as most reactive species in ROS and toxic for the cells. The hydroxyl radical is generated via the non-enzymatic Fenton reaction:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^- + {}^{\bullet}OH$$

Most cells are equipped with mechanisms that protect the vital cellular components from ROS and NOS. The ferritin superfamily proteins, which are distributed among the three domains of life in both aerobic and anaerobic organisms, play a central role by detoxifying iron by converting Fe^{2+} to Fe^{3+} through its ferroxidase activity and subsequently store Fe^{3+} as a mineral. Ferritin superfamily proteins can be categorized by the presence or absence of heme. In bacteria, the former are called ferritin-like proteins or bacterioferritins and the latter are called mini-ferritins or DNA-binding protein in starved cells (Dps). Ferritin and

bacterial ferritin-like proteins have exactly the same architecture, assembling in 24 subunits to form an inner nanocage, where a maximum of 4,500 Fe^{3+} can be sequestered as mineral after been oxidized from Fe^{2+} at the ferroxidase center [2,3]. Dps forms the dodecameric oligomer and some of them possess DNA-binding activity. It similarly oxidizes Fe^{2+} at the ferroxidase center and stores a maximum 500 Fe^{3+} inside the protein [4–6].

Mycobacterium tuberculosis complex and *Mycobacterium leprae*, the etiologic agents of tuberculosis and leprosy respectively, are a serious threat to human health. According to WHO's weekly epidemiological report in 2010, tuberculosis alone kills 1.57 million people annually and 219,826 people are suffering from leprosy. These human pathogens are obligate intracellular bacteria that can survive or multiply particularly well in macrophages and Schwann cells. These invading mycobacteria restrict the host cell iron and iron-mediated ROS generation following NADPH dependent oxidative burst as efficient strategies against host defense [7]. Mycobacteria synthesize iron-chelating molecules, siderophores, and deprive the host cells of iron; they also produce antioxidant molecules, such as superoxide dismutase and catalase. Iron overload increases the risk of tuberculosis in the African human population [8] and a deficiency in siderophore synthesis prevents the replication of *M. tuberculosis* in macrophages [9]. Thus, coordination of iron homeostasis is essential to sustain survival and growth of mycobacteria in the host [10]. Both *M. tuberculosis* and *M. leprae* produce bacterioferritins [11–13], which should be involved controlling iron homeostasis of these pathogens.

DNA is an important cellular component and living organisms should be protected from damage caused by ROS. Because *M. tuberculosis* and *M. leprae* lack DNA-binding ferritin superfamily proteins like Dps [14,15], it is thought that novel molecules that have not been identified mediate DNA protection in pathogenic mycobacteria. Colangeli et al have recently shown that mycobacterial histone-like protein Lsr2 protects DNA by acting as a physical shield [16]. However, Lsr2 lacks iron-binding and ferroxidase activities [16].

Mycobacteria including *M. tuberculosis* and *M. leprae* produce mycobacterial DNA-binding protein 1 (MDP1), also designated as laminin binding protein of *M. leprae* (ML-LBP) [17,18] (Figure S1). The N- and C-terminal halves of this protein resemble bacterial histone-like proteins, IHF and HU and eukaryotic histone H1, respectively, and possesses MDP1/ML-LBP-specific DNA-binding motif (Figure S1) [17,19–21]. In this study, we found that MDP1/ML-LBP has functions of a ferritin superfamily protein, that is both ferroxidase and iron-storage activities, and protects DNA not only physically but also prevents the iron-induced Fenton reaction. To our knowledge, this is the first report of a protein capable of storing and detoxifying iron other than ferritin superfamily proteins in living organisms.

Results

MDP1/ML-LBP possesses affinity for Fe^{3+} but not Fe^{2+}

MDP1/ML-LBP mediates several cellular processes through binding to DNA, sugar-containing molecules, and proteins both inside and outside of mycobacteria [19,21–23]. Therefore we analyzed the potential interaction between MDP1 of *Mycobacterium bovis* bacillus Calmette Guérin (BCG) (BCG-MDP1) and each ligand (analyte) by measuring surface plasmon resonance (SPR) with a BIAcore 2000 biosensor. In the course of this study, we unexpectedly observed strong increase of SPR, suggesting interaction between the analyte and immobilized ligand, when Fe^{3+} (ammonium iron (III) citrate) alone was added to the BCG-MDP1 immobilized sensor (Fig. 1A). By contrast, a gradual decrease of SPR was detected when Fe^{2+} ($FeSO_4$) was injected. Such obvious increase and decrease of SPR were not detected on injection of other metals, such as Cu^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+} (Fig. 1A).

Resonance units (RU) analyzed by BIAevaluation software of SPR are tightly correlated with the weight of bound protein ligand. However RU are not always correlated with weight in the case of protein-small molecule interaction [24]. In addition, it was reported that protein-metal interaction changes the protein structure, which in turn causes the changes of SPR [25]. Therefore, we next examined the binding capacity of BCG-MDP1 to Fe^{3+} by using radioactive ^{55}Fe. The results showed that ^{55}Fe bound to BCG-MDP1 but not to BSA coated on the ELISA plate (Fig. 1B). By contrast, iron pre-incubated with 10 mM ascorbic acid, which reduces Fe^{3+} to Fe^{2+}, did not interact with BCG-MDP1. In addition, the BCG-MDP1-^{55}Fe interaction was inhibited in the presence of excess amounts of cold Fe^{3+} but not Cu^{2+} ($CuSO_4$) (Fig. 1C). Taken together, these data demonstrate that BCG-MDP1 binds Fe^{3+}.

In order to determine whether the close mycobacterial MDP1-homologues have similar binding activity, we cloned the gene encoding MDP1 from *M. tuberculosis* H37Rv and purified it as a recombinant histidine-tagged protein (Mtb-MDP1) after expression in *E. coli*. Similarly, we also obtained recombinant ML-LBP of *M. leprae* after purification from *E. coli* expressing ML-LBP as described previously [18]. We analyzed the ^{55}Fe binding activities of these recombinant proteins. Like BCG-MDP1, both Mtb-MDP1 and ML-LBP bound to Fe^{3+} but not Fe^{2+} (Fig. 1D).

We next examined how many iron atoms MDP1 chelates using ICP-MS. BCG-MDP1 retained in the heparin column was incubated in the presence of 1 mM Fe^{3+} solution for 30 min and unbound iron was extensively washed. The BCG-MDP1-iron complex was eluted by 2 M NaCl and the iron content in the solution was determined by ICP-MS. The calculated data showed that one BCG-MDP1 contained 81.4±19.1 iron atoms (Table 1).

MDP1/ML-LBP has ferroxidase activity

The SPR analysis showed reduction of RU when Fe^{2+} was injected into a BCG-MDP1-immobilized sensor (Fig. 1A), implying some unknown responses of MDP1 in the presence of Fe^{2+}. Although MDP1 has no motifs for ferroxidase, we next examined whether MDP1/ML-LBP has enzymatic ferroxidase bioactivity.

We performed spectral analysis at 305 nm, because ferroxidase activity is the reaction, which converts Fe^{2+} to Fe^{3+}, resulting in production of the dinuclear iron (μ-oxo-bridged Fe^{3+} dimmers). To analyze the activity, protein at a concentration of 1.4 μM was incubated at 37°C for 120 seconds in 20 mM Tris-HCl (pH 7.0). Both BCG-MDP1 and buffer alone (0.4 mM $FeSO_4$) did not absorb at 305 nm during 120 seconds incubation (Fig. 2A, broken line and dush-dotted line, respectively). In contrast, in the presence of both BCG-MDP1 and 0.4 mM $FeSO_4$ there was a rapid increase in the absorbance (Fig. 2A, solid line) with a K_m value of 0.292 mM (Fig. 3A). Similarly, the enzymatic activity of both Mtb-MDP1 and ML-LBP possess identical ferroxidase activities (Fig. 2B and 2C), of which K_m values were calculated as 0.252 mM and 0.129 mM, respectively (Fig. 3B and C). By contrast, BSA and bovine histone H1 did not show such an increase in absorbance at 305 nm (Figure S2).

MDP1/ML-LBP prevents the iron-induced Fenton reaction

Since ferroxidase activity can prevent the Fenton reaction by converting Fe^{2+} into Fe^{3+}, we examined whether MDP1 and ML-LBP could abolish the Fenton reaction. Hydroxyl radical

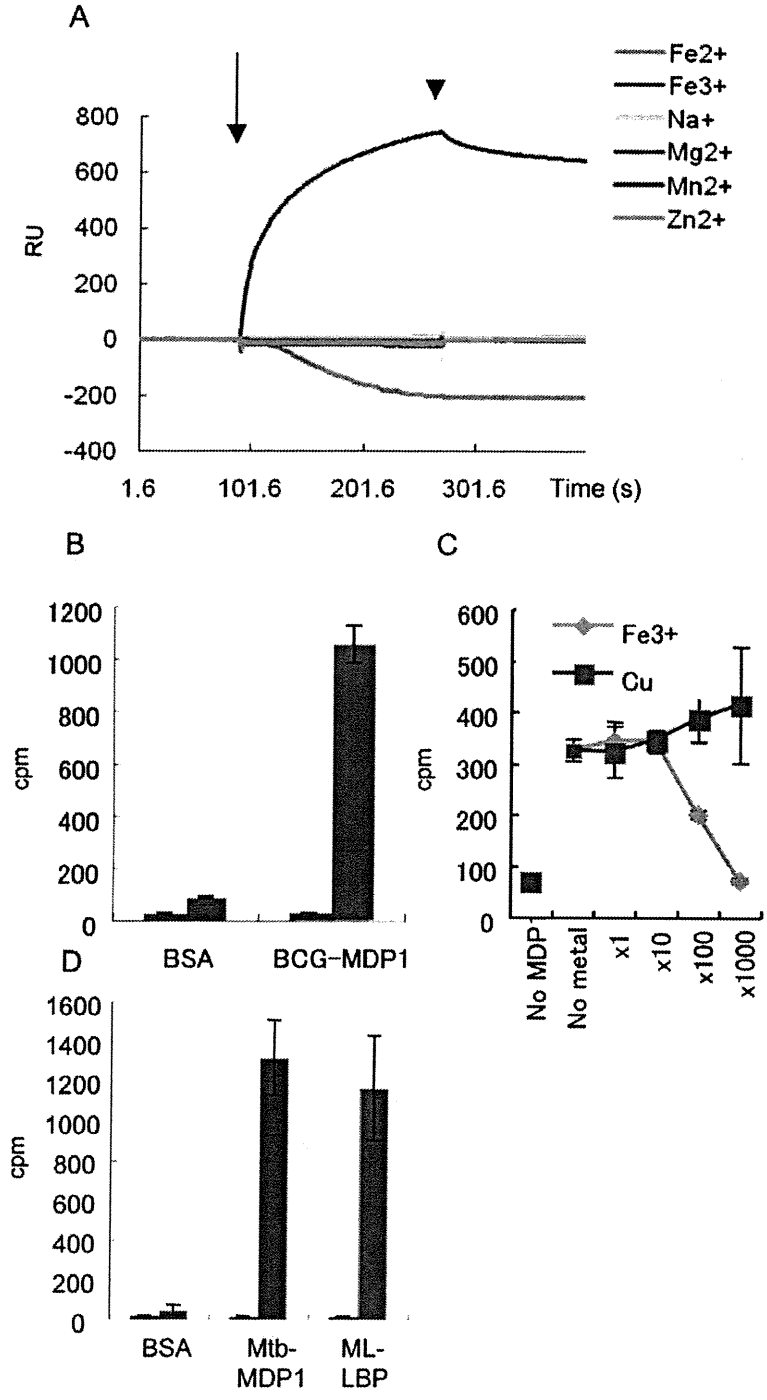

Figure 1. MDP1 binds to Fe^{3+}. (A) SPR analysis by Biacore biosensor. Each metal (1 mM) was loaded into the sensor, where BCG-MDP1 was immobilized. The SPR responses (RU, resonance units) subtracted from the MDP1-free sensor are presented. Arrow, starting point of injection of metals. Arrow head, stopping point of metal injection. Horizontal axis, time (seconds). (B) Protein-^{55}Fe interaction. Bovine serum albumin (BSA) or BCG-MDP1 was coated on a 96-well plastic plate. One μCi of $^{55}FeCl_3$ was added to each well with (green bars) or without (red bars) incubation in 10 mM ascorbic acid. The level of bound iron was counted using a scintillation counter. Vertical axis, cpm (counts per minutes) of ^{55}Fe. (C) Inhibition of MDP1-^{55}Fe interaction by cold Fe^{3+}. One μCi of $^{55}FeCl_3$ was added to the BCG-MDP1 coated or non-coated wells in the presence or absence of various molar concentrations of Fe^{3+} or Cu^{2+} as indicated. Vertical axis, level of bound ^{55}Fe. (D) Protein-^{55}Fe interaction. BSA, Mtb-MDP1, or ML-LBP was coated on a 96-well plastic plate. One μCi of $^{55}FeCl_3$ was added to each well with (green bars) or without (red bars) incubation in 10 mM ascorbic acid. Levels of bound iron were counted using a scintillation counter.
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Table 1. Iron contents determined by ICP-MS.

Samples	(μg/kg)	Iron atoms/protein
BCG-MDP1	90.0	0.6
Buffer alone	155.0	ND
Iron-loaded BCG-MDP1	10,660.0±2505.6	81.4±19.1
Iron-loaded Mtb-MDP1	18,166.7±138.7	138.7±35.5

ND, not detected.
doi:10.1371/journal.pone.0020985.t001

generated in the presence of 1 mM H_2O_2 and 25 or 50 μM $FeSO_4$ by the Fenton reaction was monitored by "8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4-(2H,3H) dione" (L-012), which reacts with reactive oxygen species and develops strong chemiluminescence (CHL) [26]. Hydroxyl radical was generated depending on the concentration of Fe^{2+} (Fig. 4A). In this experimental setting, the addition of 3 μM BCG-MDP1 remarkably suppressed the generation of hydroxyl radical (Fig. 4A). Furthermore, the addition of BCG-MDP1 after initiation of the Fenton reaction suppressed the production of hydroxyl radical even under optimal generating conditions (Fig. 4B). Similarly, both Mtb-MDP1 and ML-LBP suppressed the generation of hydroxyl radical by the Fenton reaction (Fig. 5). In contrast, neither histone H1 nor 3 μM BSA suppressed the Fenton reaction (Fig. 4A & B). Furthermore, the suppressive activity of 3 μM Mtb-MDP1 or ML-LBP was equivalent to 30 μM of the antioxidant sugar, ascorbic acid (data not shown).

Hydroxyl radical can be generated in the presence of bivalent metals other than iron and H_2O_2. In order to clarify whether the suppressive effect on the Fenton reaction by MDP1 is dependent on its ferroxidase activity, we studied the effects of BCG-MDP1 on Cu^{2+}-dependent Fenton-like reactions. For these studies hydroxyl radical was generated in the presence of 25 or 50 μM $CuSO_4$ and 1 mM H_2O_2 (Fig. 4C). The data showed that neither MDP1 nor histone H1 suppressed Cu^{2+}-induced Fenton-like reactions, but in fact enhanced the reaction. These data reinforce the hypothesis that inhibition of iron-induced Fenton reactions by MDP1 is indeed dependent on ferroxidase activity.

Dual mechanism of DNA protection by MDP1/ML-LBP

We next examined the effect of MDP1 on the protection of DNA. First, plasmid DNA (pUC19) was incubated with DNase1 in the presence or absence of 3 μM BCG-MDP1 and histone H1. We found that DNA was protected equally by MDP1 and histone H1 (Fig. 6A). Thus, both MDP1 and histone H1 protect DNA from DNase1 by acting as physical shields.

Comparison of the effects of MDP1 and histone H1 on DNA damage induced by the Fenton reaction revealed that DNA was degraded in the presence of 25 or 50 μM $FeSO_4$ and 1 mM H_2O_2 (Fig. 6B). BCG-MDP1 protected DNA from degradation by the Fenton reaction, whereas the level of protection exerted by histone H1 was comparatively lower (Fig. 6B). By contrast, neither BCG-MDP1 nor histone H1 could protect DNA from Cu^{2+}-induced Fenton-like reactions (Fig. 6C), further suggesting that MDP1 protects DNA by suppressing iron-induced Fenton reactions mediated through its ferroxidase activity. As expected both Mtb-MDP1 and ML-LBP also protected DNA from digestion with DNase1 and the damage caused by the Fenton reaction in a similar manner to BCG-MDP1 (Fig. 6D). Taken together, these data suggest that MDP1 and ML-LBP protect DNA in a dual fashion, by acting as a physical barrier and minimizing the Fenton reaction.

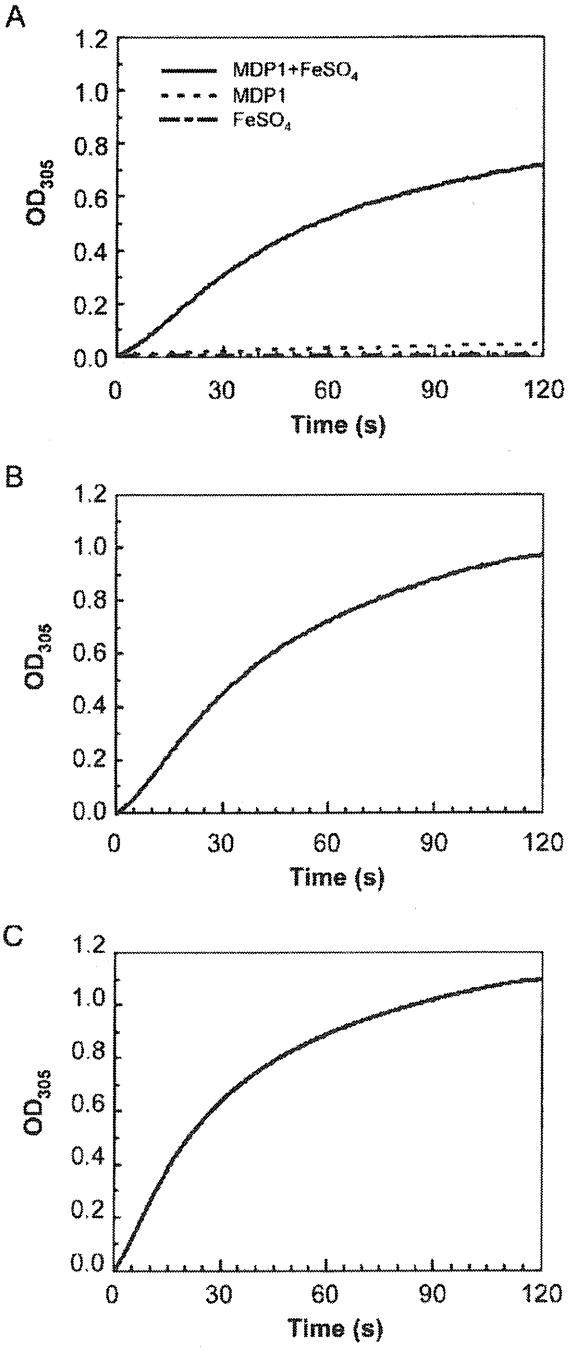

Figure 2. Ferroxidase activity of MDP1/ML-LBP. The conversion from Fe^{2+} to Fe^{3+} was determined by measuring the absorbance at 305 nm. (A) broken line, 1.4 μM BCG-MDP1; dush-dotted line, 0.4 mM $FeSO_4$; solid line, 0.4 mM $FeSO_4$+1.4 μM BCG-MDP1. (B) solid line, 0.4 mM $FeSO_4$+1.4 μM Mtb-MDP1. (C) solid line, 0.4 mM $FeSO_4$+1.4 μM ML-LBP. The reaction was monitored for 120 seconds.
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The role of MDP1 in the detoxication of iron in *Mycobacterium*

Finally, we assessed the role of MDP1 in the detoxication of iron in *Mycobacterium* itself. MDP1 is presumed to be essential for slow

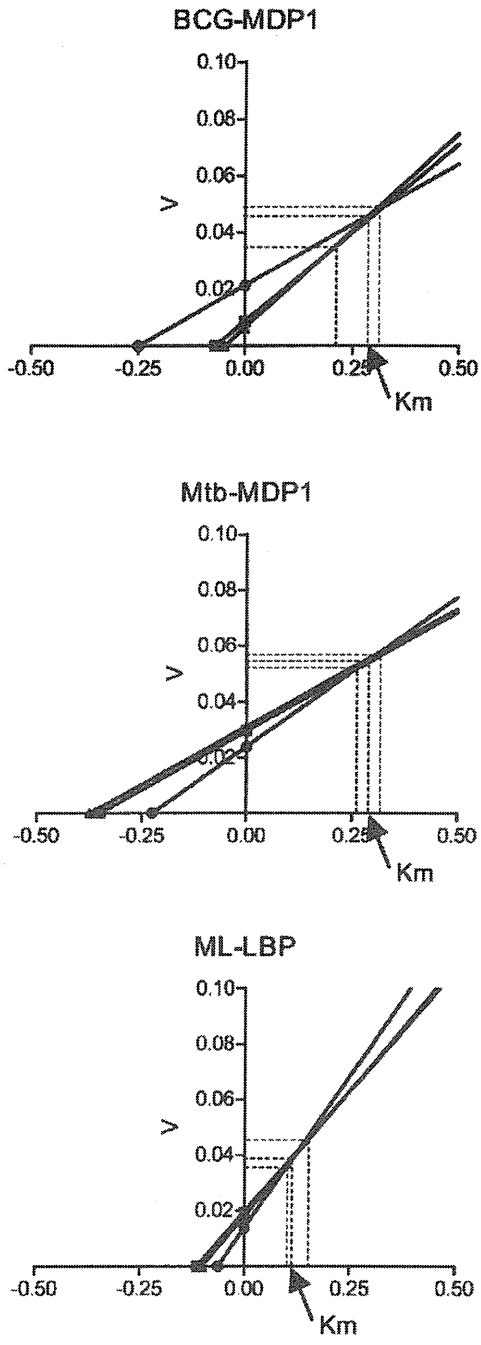

Figure 3. Ferroxidase activity of BCG-MDP1, Mtb-MDP1, and ML-LBP. The conversion from Fe^{2+} to Fe^{3+} was monitored by spectral analysis at 305 nm. 0.0125–0.1 mM $FeSO_4$ were added to 1.4 μM BCG-MDP1 (A), Mtb-MDP1 (B) and ML-LBP (C). Direct linear plots were shown by the straight lines intercepting X-axis with $-1/K_m$. K_m are taken as the median value from each series.
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growers of mycobacteria, such as BCG and *M. tuberculosis* [27,28] and cannot be knocked out. Therefore we employed the MDP1-homologue (Ms-MDP1)-deficient strain of rapid grower *Mycobacterium smegmatis* [29]. We detected the ferroxidase activity of purified Ms-MDP1, of which the K_m value was 0.136 mM (Figure S2). We compared the resistance to H_2O_2 among wild type and mutant strains of *M. smegmatis*, including Ms-MDP1-deficient, and Ms-MDP1-complemented strains. These strains were treated with 12.5 mM H_2O_2 in the logarithmic phase of growth and the survival rate was determined by a colony forming unit (CFU) assay. There was a 100-fold reduction in survival of the Ms-MDP1-deficient strain (Fig. 7). We also pretreated bacteria with 40 mM desferal, an iron chelator. This treatment remarkably inhibited the bactericidal effect of H_2O_2 (Fig. 7), showing that major bactericidal effect with H_2O_2 treatment is depending on the Fenton reaction. Together, our data show that MDP1 play a role in iron detoxfication and bacterial survival.

Phylogenetic analysis of MDP1/ML-LBP homologues and ferritin-superfamily proteins

We accomplished molecular evolutionary analysis to recognize the phylogenetic diversity of MDP1/ML-LBP homologues and ferritin-superfamily proteins. We constructed phylogenetic dendrogram of MDP1/ML-LBP homologues and ferritin-superfamily proteins (bacterioferritin and Dps) of mycobacteria, such as BCG, *M. tuberculosis*, *Mycobacterium avium*, *Mycobacterium avium* subsp. *paratuberculosis*, *M. smegmatis*, and *M. leprae*. We also applied amino acid sequences of other bacterial ferritin-superfamily proteins of *E. coli*, *Salmonella enterica*, *Yersinia pestis*, and *Helicobacter pylori* and the dendrogram was created using clustering with the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) by GENETYX software (Genetyx, Tokyo, Japan). In the phylogenetic tree (Figure 8), MDP1/ML-LBP homologues formed different cluster from ferritin-superfamily proteins, supporting phylogenetic distinctiveness between MDP1/ML-LBP homologues and ferritin superfamily proteins.

Discussion

Iron in living organisms is a double-edged sword, as it is an essential and beneficial element but is also harmful. Therefore, living organisms strictly control iron homeostasis. The ferritin superfamily proteins are distributed across all three domains of life and contribute to iron detoxication through oxidation and storage as Fe^{3+}, the nontoxic form of iron. Bacteria produce two types of ferritin superfamily proteins, one is bacterioferritin and another is Dps (also called miniferritin). Bacterioferritin is distributed among mycobacteria [12,13,30–32] but pathogenic mycobacteria, such as *M. tuberculosis* complex and *M leprae*, lack DNA-binding ferritin superfamily proteins like Dps. Here we show a histone-like mycobacterial protein, MDP1/ML-LBP has similar activity with ferritin superfamily proteins and protect DNA by preventing the Fenton reaction.

MDP1/ML-LBP is a major cellular component of mycobacterial cell [17,18] and is a multifunctional molecule depending on interaction with biomolecules, such as DNA, laminin [18,33], glycosaminoglycans [19,33], and glycolipids [22,28], and in turn controls gene expression, infection, and cell wall integrity [19,22,23,28,33]. In contrast, in this study, we identified enzymatic activity of MDP1 itself that should be involved in iron-homeostasis of mycobacteria.

This study was initiated by the unexpected detection of the increase and decrease of SPR, when Fe^{3+} and Fe^{2+} were loaded into BCG-MDP1-immobilized sensors, respectively (Fig. 1A). Because altered protein structure changes RU of SPR [25], we examined whether MDP1 actually binds to iron. We have shown that BCG-MDP1 binds to Fe^{3+}, but not to Fe^{2+} (Fig. 1B and C) and captures 81.4±19.1 iron atoms/protein, as measured by ICP-MS (Table 1). We also confirmed similar iron-binding activity of recombinant Mtb-MDP1 and ML-LBP (Fig. 1D). MDP1 is eluted in the 150–

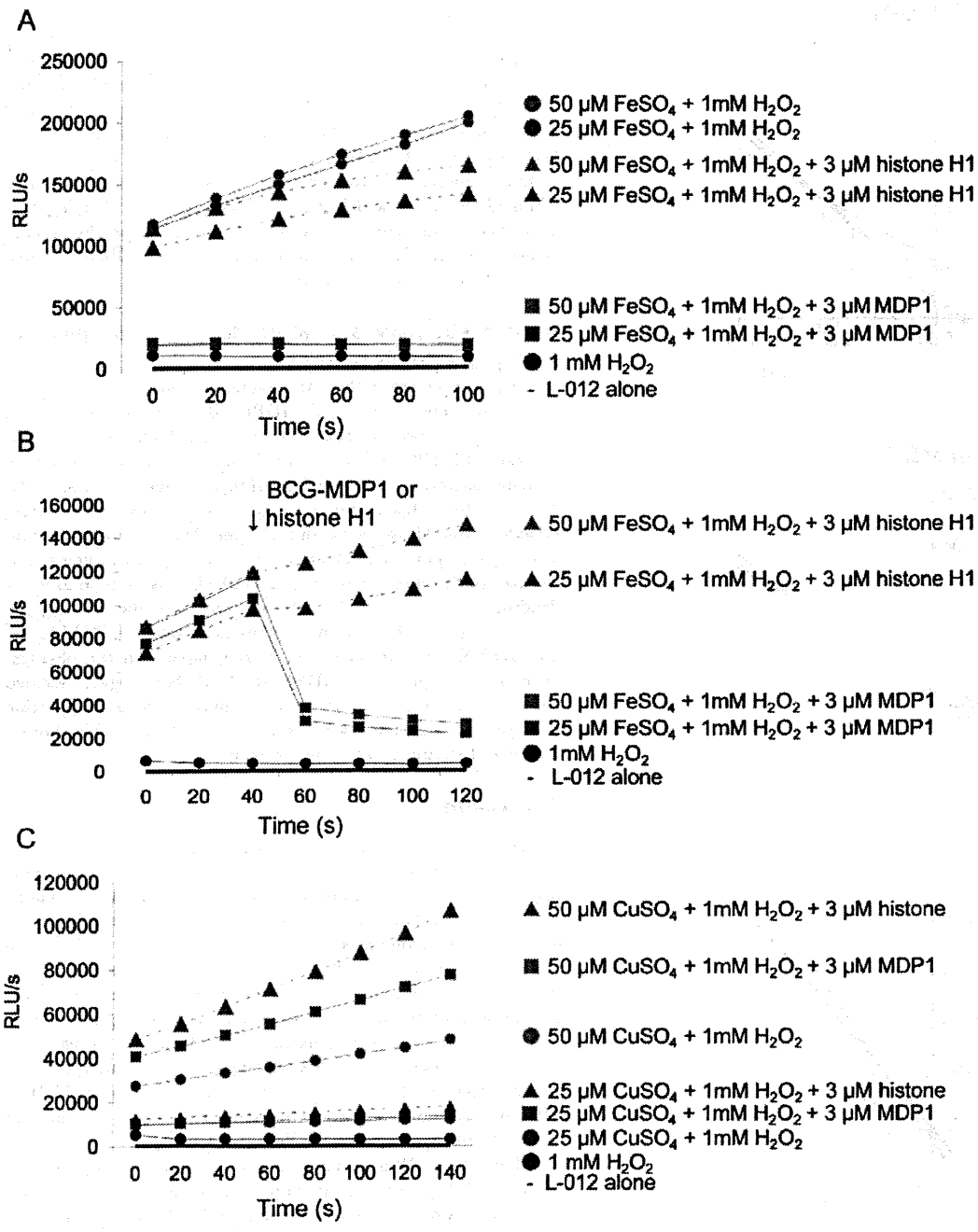

Figure 4. MDP1 suppresses the generation of the hydroxyl radical by the Fenton reaction. The hydroxyl radical level was measured by a L-012 probe sensitive to oxygen radicals. L-012 probe was added in all samples. RLU, relative luminescence units. (A) Proteins were added at the initiating time point of the Fenton reaction by H_2O_2 and $FeSO_4$. (B) Proteins were added 40 seconds after initiating the Fenton reaction. (C) The effect of proteins on the Fenton like reaction generated by H_2O_2 and $CuSO_4$.
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210 kDa fraction by gel filtration [17], suggesting that 7–10 MDP1 subunits oligomerize. Thus, it is estimated that oligomerized MDP1 can sequester 532–760 iron molecules, which is comparable to the iron-storage activity of ferritin superfamily proteins. The reduction of RU of SPR, when Fe^{2+} was injected into MDP1-immobilized sensor might be due to structural change of MDP1.

MDP1/ML-LBP is phylogenetically distinctive protein from ferritin superfamily proteins as shown in Figure 8. A remarkable difference in the method of iron storage between MDP1 and ferritin is that MDP1 directly captures Fe^{3+}, while ferritin incorporates iron after oxidizing Fe^{2+} using oxygen or hydrogen peroxide at the ferroxidase center. Thus, ferroxidase activity is dispensable for iron sequestration by MDP1/ML-LBP but not by ferritin superfamily proteins. These data suggest that MDP1/ML-LBP is a new type of iron detoxication and storage protein. The ferroxidase activity of MDP1 using O_2 as oxidant was slightly