イソニアジド抵抗性を付与する。カタラーゼは、本来呼吸で生じる過酸化水素を、不均化によって酸素と水に分解する酵素である。前節で述べたように、MDP1は2価鉄の存在下で過酸化水素を水にするという結果的に同様の活性を有していることが、結核菌内でKatGと相反して発現が調節され、それが結果的に潜在期のイソニアジド抵抗性を付与しているものと推測される。

おわりに

結核を筆頭として、ハンセン病、非結核性抗酸菌症、動物でのヨーネ病など、抗酸菌感染症の脅威は継続している。これら病原性抗酸菌は、一般の細菌と異なり、遅発育性で、一部は休眠して人類を含む宿主に寄生し、薬剤感受性の低下をもたらす。本稿では、遅発育性や薬剤抵抗性に関わる抗酸菌分子についての私たちのこれまでの研究成果を紹介したが、病原性抗酸菌の特異な生存戦略の解明が、病原体のアキレス腱を暴き、革新的な感染制御法の構築につながることを期待する。

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Molecular mechanisms of dormancy and drug tolerance in mycobacteria

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Key words : iron, dormancy, drug resistance, LTBI, endogenous reactivation

Instead of rapid multiplication, pathogenic mycobacteria, such as *Mycobacterium tuberculosis* are likely to have acquired slow but long life. Host immunity affords desirable non-competitive environment for *M. tuberculosis* in human lungs, where this pathogen slowly grows or arrests growing, which avoids rapid loss of living places. Mycobacterial DNA-binding protein 1 (MDP1), a unique histone-like protein associating mycobacterial GC-rich DNA, has pivotal role in realizing such slow life and pathogenesis including drug tolerance to isoniazid.

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Th1-skewed tissue responses to a mycolyl glycolipid in mycobacteria-infected rhesus macaques

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ABSTRACT

Trehalose 6,6′-dimycolate (TDM) is a major glycolipid of the cell wall of mycobacteria with remarkable adjuvant functions. To avoid detection by the host innate immune system, invading mycobacteria down-regulate the expression of TDM by utilizing host-derived glucose as a competitive substrate for their mycolyltransferases; however, this enzymatic reaction results in the concomitant biosynthesis of glucose monomycolate (GMM) which is recognized by the acquired immune system. GMM-specific, CD1-restricted T cell responses have been detected in the peripheral blood of infected human subjects and monkeys as well as in secondary lymphoid organs of small animals, such as guinea pigs and human CD1-transgenic mice. Nevertheless, it remains to be determined how tissues respond at the site where GMM is produced. Here we found that rhesus macaques vaccinated with *Mycobacterium bovis* bacillus Calmette–Guerin mounted a chemokine response in GMM-challenged skin that was favorable for recruiting T helper (Th)1 T cells. Indeed, the expression of interferon-γ, but not Th2 or Th17 cytokines, was prominent in the GMM-injected tissue. The GMM-elicited tissue response was also associated with the expression of monocyte/macrophage-attracting CC chemokines, such as CCL2, CCL4 and CCL8. Furthermore, the skin response to GMM involved the up-regulated expression of granulysin and perforin. Given that GMM is produced primarily by pathogenic mycobacteria proliferating within the host, the Th1-skewed tissue response to GMM may function efficiently at the site of infection.

1. Introduction

Mycobacteria possess highly lipid-rich cell walls that are critical not simply for their acid-fast properties but also for their pathogenesis. Outside the peptidoglycan structure, mycobacteria-specific, long-chain fatty acids, referred to as mycolic acids, are aligned densely in covalent association with the 6-position of arabinose termini of the underlying arabinogalactan sugar layer, thereby forming the rigid skeleton of the cell wall [1]. Mycolic acids also exist at the surface of the cell wall as free molecules complexed to sugars. The extremely hydrophobic cell wall architecture constructed by interactions between the arabinogalactan-linked mycolic acids and carbon chains of the surface-exposed glycolipids is essential for protection from a variety of chemical agents, such as reactive oxygen intermediates and hydrolytic enzymes, derived from the host cells [2].

Among mycolyl glycolipids, trehalose 6,6′-dimycolate (TDM) has been studied extensively because of its abundance in the cell wall of mycobacteria and its potent adjuvancy [3]. TDM is recognized by host innate immune receptors, including the macrophage-inducible C-type lectin (Mincle), and strongly activates macrophages to secrete proinflammatory cytokines [4]. However, pathogenic mycobacteria appear to have evolved an evasive maneuver to down-regulate TDM expression to avoid unnecessary stimulation of the host innate immunity. Mycobacteria-derived mycolyltransferases catalyze the final step of TDM biosynthesis, using trehalose 6-monomycolate as a substrate, but upon entry into the host, mycobacteria utilize host-derived glucose as a competitive substrate for the enzymes, resulting in down-regulation of TDM expression and up-regulated production of glucose monomycolate (GMM) [5]. As glucose is present at high concentrations in the host and scarce in external environments, only mycobacteria

Abbreviations: Ag, antigen; BCG, *Mycobacterium bovis* bacillus Calmette–Guerin; GMM, glucose monomycolate; TDM, trehalose 6,6′-dimycolate.

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that sustain reasonable metabolism within the host, not those that are killed by the host or viable only in environments, can produce GMM. Thus, GMM is regarded as a marker glycolipid for pathogenic mycobacteria that actively proliferate within the host.

Notably, the acquired immune system is equipped with T cells that react to GMM specifically. A GMM-specific, CD1b-restricted T cell line was established from a patient with leprosy [6], and GMM-specific, CD1c-restricted T cell lines were obtained from *Mycobacterium bovis* bacillus Calmette–Guerin (BCG)-vaccinated rhesus macaques [7]. In addition, T cell responses to GMM in the peripheral blood, lymph nodes, and spleen have been detected in humans [8], rhesus macaques [7], cattle [9], guinea pigs [10], and human CD1-transgenic mice [11]. However, these studies, including ours [7,10], did not address directly how tissues responded at the site where GMM production occurred. Therefore, the present study was designed to determine the quality of the tissue response elicited at the site challenged with GMM, using BCG-vaccinated rhesus macaques. We found that GMM provoked highly Th1-skewed local responses.

2. Materials and methods

2.1. Animals and vaccination

The rhesus macaques (*Macaca mulatta*) used in this study were treated humanely in accordance with institutional regulations, and experimental protocols were approved by the Committee for Experimental Use of Non-human Primates at the Institute for Virus Research, Kyoto University. Vaccination with the Tokyo 172 strain of BCG (Japan BCG Laboratory, Tokyo, Japan) was performed as described previously [7].

2.2. Purification of GMM and preparation of liposomes

GMM was purified from cultured mycobacteria and integrated into stearylated octaarginine-containing liposomes as described previously [10]. The purity of the GMM sample was confirmed by TLC, using two different solvent systems, and its molecular identify was confirmed by mass spectrometry. Protein contamination was not detected by silver staining of SDS–PAGE gels or by the Bradford assay.

2.3. DNA microarray

Peripheral blood mononuclear cells (PBMCs) were obtained from BCG-vaccinated monkeys (MM553 and MM556) and placed in wells of 24-well tissue culture plates (6×10^6/well). The cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal calf serum (Hyclone, Logan, UT) in the presence of either GMM in liposomes (1 μg/ml) or empty liposomes. After 24 h at 37 °C, total RNA was extracted using the RNeasy mini kit (Qiagen, Hilden, Germany) and sent to HaploPharma, Inc. (Okinawa, Japan), where a DNA microarray analysis was performed as instructed by the Expression Analysis Technical Manual, 2005 (Affymetrix, Santa Clara, CA). Briefly, biotinylated complementary RNAs (cRNAs) were prepared from 250 ng of total RNA, and the cRNA fragments were hybridized for 16 h at 45 °C on the GeneChip rhesus macaque genome array (Affymetrix). The GeneChips were subsequently washed, stained with the Affymetrix Fluidics Station 450, and scanned using the Gene Chip Scanner 3000 7G. The data were analyzed with Microarray Suite version 5.0 using Affymetrix default analysis settings. The obtained microarray data were deposited in the GEO database (accession number GSE44963).

Table 1
Primers used for RT-PCR.

Targets	Primers
CCL1	5′-CAA GAC GTG GAC AGC AAG AG-3′(sense) 5′-CAT CTA GCC TGG TTC AAG GC-3′ (anti-sense)
CCL2	5′-TCT GTG CCT GCT GCT CAT AG-3′ (sense) 5′-CGG AGT TTG GAT TTG CTT GT-3′ (anti-sense)
CCL3	5′-TTG CTG TCC TCC TCT GCA C-3′ (sense) 5′-CGT ATT TCT GGA CCC ACT CC-3′ (anti-sense)
CCL4	5′-GTT CTG TAG CCT CAC CTC TG-3′ (sense) 5′-GAC TTG CTT GCC TCT TTT GG-3′ (anti-sense)
CCL7	5′-CAC CTC TGT GTC TGC TGC TC-3′ (sense) 5′-CAT GGC TTG GTT TCA GTT CA-3′ (anti-sense)
CCL8	5′-CTT CTG TGC CTG CTG CTC AT-3′ (sense) 5′-ATC CCT GAC CCA TCT CTC CT-3′ (anti-sense)
CCL11	5′-ACC TGC TGC TTT ACC CTG AC-3′ (sense) 5′-AGT TGG AGA TTT TCG GTC CA-3′ (anti-sense)
CCL17	5′-CTT CTC TGC AGC ACA TCC AT-3′ (sense) 5′-AAC AGA TGG CCT TGT TCT GG-3′ (anti-sense)
CCL24	5′-AAC CAG CCT TCT GTT CCT TG-3′ (sense) 5′-GGC ATC CAG GTT CTT CAT GT-3′ (anti-sense)
CXCL9	5′-TTT TCC TCT TGG GCA TCA TC-3′ (sense) 5′-TTT GGC TGA CCT GTT TTT CC-3′ (anti-sense)
CXCL10	5′-CAT TCT GAT TTG CTG CCT TG-3′ (sense) 5′-TTG ATG GCC TTA GAT TCT GGA-3′ (anti-sense)
CXCL11	5′-GAG TGT GAA GGG CAT GGC TA-3′ (sense) 5′-TGG GAT TTA GGC ATC GTT GT-3′ (anti-sense)
Granulysin	5′-ACC CAG AGA AGC ATT TCC AA-3′ (sense) 5′-GGG AAG GGA GAC TGG AGA GT-3′ (anti-sense)
Perforin	5′-CCC TCT GTG AAA ATG CCC TA-3′ (sense) 5′-GAT GAA GTG GGT GCC GTA GT-3′ (anti-sense)
GAPDH	5′-ACC ACA GTC CAT GCC ATC AC-3′ (sense) 5′-TCC ACC ACC CTG TTG CTG TA-3′ (anti-sense)

2.4. RT-PCR

To assess tissue responses to GMM, liposome containing 5 μg of GMM as well as an equivalent amount of empty liposome was dissolved in 100 μL of phosphate-buffered saline and injected into the skin of BCG-vaccinated monkeys. After 2 days, the monkeys were sacrificed and the excised skin was deep-frozen in liquid nitrogen. Each skin sample (approximately 100 mg) was disrupted by operating the Tomy MS-100R beads cell disruptor (Tomy Seiko Co., Tokyo, Japan) with 5 mm beads, and total RNA was extracted using the RNeasy fibrous tissue midi kit (Qiagen). The first-strand cDNA

Table 2
Primers used in this study for real time PCR.

Targets	Primers
IFNG	5′-GAC ATC TTG AGG AAT TGG AAA G-3′ (sense) 5′-TTT GGA TCC TCT GGT CAT CTT-3′ (anti-sense)
T-bet	5′-CAC CTG TTG TGG TCC AAG TTT-3′ (sense) 5′-TGA CAG GAA TGG GAA CAT CC-3′ (anti-sense)
IL10	5′-TGC CTT CAG CAG AGT GAA GA-3′ (sense) 5′-GCA ACC CAG GTA ACC CTT AAA-3′ (anti-sense)
GATA3	5′-ACT ACG GAA ACT CGG TCA GG-3′ (sense) 5′-GGC AGG GAT CCA TGA AGC AG-3′ (anti-sense)
IL17F	5′-TGG GAA GAC CTC ATT GGT GC-3′ (sense) 5′-GGA TTT CGT GGG ATT GCT AT-3′ (anti-sense)
RORγt	5′-CAG CGC TCC AAC ATC TTC T-3′ (sense) 5′-CAC AGC GTT CCC ACA TCT C-3′ (anti-sense)
CD3E	5′-AGA TGC AGT CGG GCA CTC-3′ (sense) 5′-TAC CAT CTT GCC CCC AAA C-3′ (anti-sense)

Table 3
Chemokines up-regulated in GMM-stimulated PBMCs.[a]

Genes	Probe set ID	MM553	MM556
Cytokines			
IFNG	MmuSTS.173.1.S1_at	4.9	7.8
IFNG	MmugDNA.41414.1.S1_at	6.6	5.4
IL26	MmuSTS.4352.1.S1_at	2.4	4.9
IL6	Mmu.12240.1.S1_at	3.8	4.2
IL6	MmuSTS.4354.1.S1_at	4.3	3.3
LTA	MmuSTS.1446.1.S1_at	2.2	3.9
C chemokines			
None			
CC chemokines			
CCL2 (MCP-1)	Mmu.11912.1.S1_at	18.4	4.8
	MmuSTS.3317.1.S1_at	2.1	14.6
CCL8 (MCP-2)	MmugDNA.3158.1.S1_at	15.5	3.6
CCL7 (MCP-3)	MmuSTS.3575.1.S1_at	3.5	11.0
CCL3 (MIP-1α)	Mmu.6471.1.S1_at	3.8	2.7
	Mmu.6471.1.S1_x_at	2.5	3.7
CXC chemokines			
CXCL9	Mmu.11358.1.S1_at	93.1	8.4
	MmuSTS.4003.1.S1_at	88.5	3.3
CXCL10	Mmu.11363.1.S1_at	17.8	2.1
CXCL11	Mmu.11366.1.S1_at	57.0	3.6
	MmugDNA.15618.1.S1_s_at	44.2	2.8
	MmugDNA.19126.1.S1_at	37.9	2.2
CX3C chemokines			
None			

[a] Values indicate the fold increase in transcription levels after GMM stimulation.

was synthesized from 1 μg of RNA using oligo(dT) and the PrimeScript reverse transcriptase (Takara Bio, Inc., Otsu, Japan). The samples were then subjected to PCR for 25 s at 94 °C, 30 s at 58 °C, and 45 s at 72 °C using Taq DNA polymerase. The primers used are listed in Table 1.

2.5. Real-time PCR

PCRs were performed using the THUNDERBIRD SYBR qPCR Mix (Toyobo, Osaka, Japan) according to the manufacturer's instructions. Briefly, a reaction mixture (20 μL) containing 0.4 μL of each cDNA sample, 10 μL of the THUNDERBIRD SYBR qPCR Mix, 0.4 μL of the ROX reference dye, and 0.6 μm of each primers was subjected to PCR using the Applied Biosystems 7500 real-time PCR system. The reactions were run in triplicate samples, and the absolute mean values for each gene were normalized to that of the CD3ε gene. The primers used are listed in Table 2.

2.6. Histochemistry

The skin samples were fixed for 1 day with 4% paraformaldehyde, dehydrated, and deep-frozen in OCT compound. The cryosections were stained with hematoxylin and eosin and observed under a microscope. Some cryosections were processed for immunohistochemistry as described previously [12], and labeled with an anti-rhesus macaque CD14 monoclonal antibody (MF396) generated in our laboratory, or a negative control antibody (RPC5.4) [13]. Signals were visualized with the standard protocol using a Vector Elite ABC kit.

2.7. Statistics

Statistical analysis was performed, using the one-way Analysis of Variance (ANOVA).

3. Results and discussion

3.1. Peripheral blood cytokine and chemokine responses to GMM

Cytokines and chemokines play a pivotal role in determining the quality of immune responses in tissues. We assumed that cytokine/chemokine profiles detected for GMM-stimulated PBMCs might provide a clue to tissue responses to GMM. Therefore, to grasp an overview of these responses, we performed a DNA microarray analysis, using RNA derived from GMM-stimulated PBMCs. PBMCs were isolated from 2 BCG-vaccinated monkeys (MM553 and MM556) that contained a significant pool of GMM-specific circulating T cells and stimulated in vitro with either the GMM liposome or empty liposome. After 24 h, total RNA was extracted from the cells and subjected to the GeneChip analysis. On the basis of the microarray data (GEO database accession number, GSE44963), transcriptional levels for each of the known 59 cytokines and 40 chemokines were analyzed, and those with more than a 2-fold increase in both monkeys in response to GMM were listed. As shown in Table 3, the listed cytokines included Th1 cytokines (IFNG and LTA) and a proinflammatory cytokine (IL6), but the up-regulated expression of representative Th2 cytokines, such as IL4 and IL10, was not observed. In addition, the up-regulation of Th17-related cytokines, such as IL17A, IL17F, IL21, and IL22, was

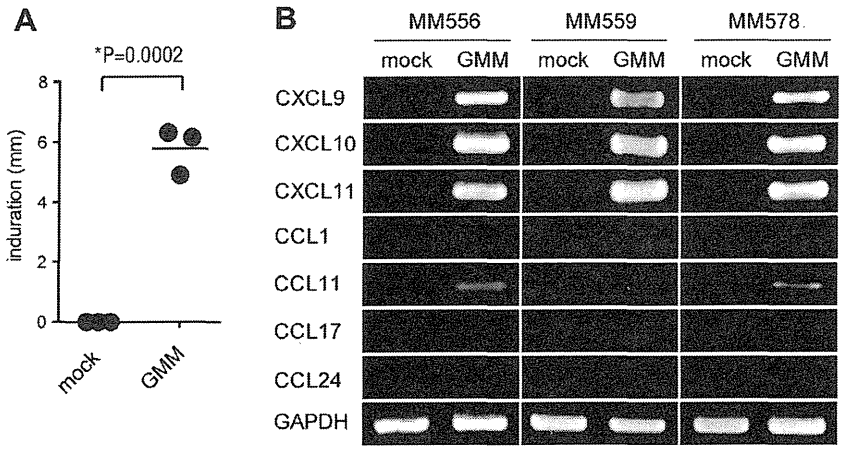

Fig. 1. Up-regulated expression of Th1-attracting chemokines in GMM-challenged skin. (A) Three BCG-vaccinated rhesus macaques (MM556, MM559, and MM578) received an intradermal injection of either the GMM liposome (GMM) or empty liposome (mock). After 2 days, the skin induration at the site of injection was measured. (B) RNA was extracted from the skin, and RT-PCR was performed for detection of chemokine expression.

not apparent except for IL26, a potential Th17 cell-associated cytokine. In parallel with these cytokine responses, skewed chemokine responses were also indicated. Besides monocyte chemotactic proteins (MCPs), such as CCL2, CCL7, CCL8, and macrophage inflammatory protein (MIP)-1α (CCL3), the expression of CXCL9, CXCL10, and CXCL11, a typical set of CXC chemokines with the potential to attract Th1-type T cells, was prominently up-regulated by stimulation with GMM. On the basis of these microarray data, we predicted that the tissue response to GMM may involve the expression of Th1 cytokines and chemokines. To address this directly, tissue responses to GMM were evoked in the skin of BCG-vaccinated rhesus macaques and analyzed for their cytokine/chemokine expression profiles.

3.2. GMM-induced tissue chemokine responses

Our previous study [7] indicated that GMM-specific T cell responses could be detected in BCG-vaccinated, but not unvaccinated rhesus macaques. Therefore, liposome containing 5 μg of GMM as well as an equivalent amount of empty liposome was injected separately into the skin of 3 BCG-vaccinated rhesus macaques (MM556, MM559, and MM578). After 2 days, significant skin induration was induced at the site of GMM challenge in all 3 animals whereas no apparent response was elicited toward empty liposome (Fig. 1A). The skin was excised and total RNA was extracted, followed by RT-PCR for expression of representative T cell-attracting chemokines. As shown in Fig. 1B, CXCL9, CXCL10, and CXCL11, a group of chemokines known to preferentially attract Th1 cells [14], were highly transcribed in the GMM-injected tissue. In sharp contrast, transcription of Th2-attracting chemokines [15], such as CCL1, CCL11, CCL17, and CCL24, was either marginal (for CCL11) or barely detectable (for CCL1, CCL17, CCL24) (Fig. 1B). Therefore, the predominant expression of the Th1-attracting chemokines over the Th2-attracting chemokines predicts a Th1-skewed local response in the GMM-injected skin.

3.3. IFN-γ dominant tissue responses to GMM

Hematoxylin and eosin staining of the GMM-challenged skin of the MM556 rhesus macaque revealed prominent infiltration by mononuclear cells (Fig. 2A, right), which was not observed in the mock-challenged skin (left). In order to assess the net cytokine response at the site of the antigen (Ag) challenge, total RNA was extracted from the GMM-challenged and mock-challenged skin of the 3 rhesus macaques, and transcriptional levels for hallmark cytokines, such as IFNG (for Th1), IL10 (for Th2), and IL17 (for Th17), were determined by real-time PCR. As shown in Fig. 2B, IFNG expression was significantly up-regulated in response to GMM ($P = 0.0002$) whereas no up-regulation was observed for IL10 and IL17. In parallel with the skewed cytokine response, the expression of T-bet, a Th1 cell-specific transcription factor that controls IFNG expression, appeared to be up-regulated in the GMM-challenged skin although the increase was not statistically significant ($P = 0.066$). In sharp contrast, the expression of GATA-3, a Th2-specific transcription factor, and RORγt, a transcription factor promoting T cell differentiation into Th17 cells [16], was decreased significantly ($P = 0.021$ and $P = 0.046$, respectively) (Fig. 2B). Taken together, these results pointed to the IFN-γ dominant response in the GMM-challenged skin.

Our previous study indicated that the GMM-specific T cell population developed in BCG-vaccinated rhesus macaques contained $CD8^+$ cytotoxic T cells [7], and therefore, we predicted that the expression of cytotoxic granule proteins, such as granulysin and perforin, might be up-regulated in the GMM-challenged skin. Indeed, we found that the transcription of the granulysin and perforin genes was readily detectable by RT-PCR in the GMM-challenged, but not mock-challenged skin of the 3 rhesus macaques analyzed (Fig. 2C).

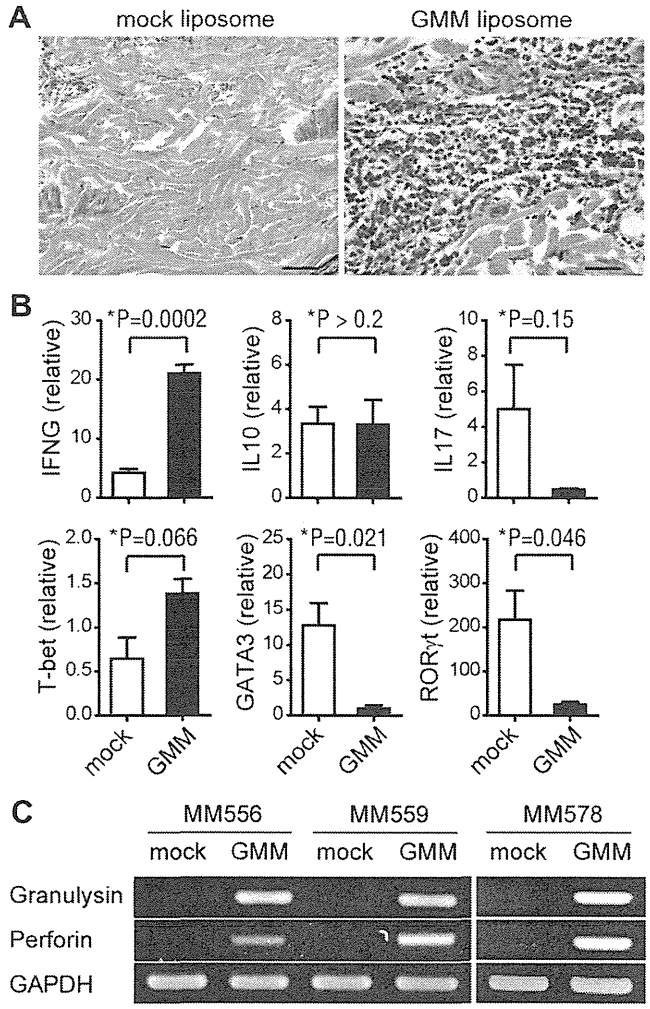

Fig. 2. Th1-skewed responses in the GMM-challenged skin. (A) The skin sections derived from MM556 were stained with hematoxylin and eosin, and viewed under a microscope. Scale bars, 50 μm. (B) Gene expression of cytokines and related transcription factors was analyzed by quantitative real-time PCR, using RNA extracted from the GMM-challenged and mock-challenged skin. The absolute values for each gene were normalized to that of the *CD3ε* gene. Statistical assessment of the real time data obtained form the 3 animals was performed. (C) RNA was extracted from the GMM liposome-challenged or mock-challenged skin as in Fig. 1, and RT-PCR was performed for transcription of the granulysin and perforin genes.

3.4. Macrophage recruitment

IFN-γ orchestrates the trafficking of specific immune cells, including monocytes and macrophages, to sites of inflammation through up-regulating expression of a group of chemokines. Indeed, an immunohistochemical analysis of the GMM-challenged skin with an anti-rhesus macaque CD14 monoclonal antibody detected a local accumulation of $CD14^+$ mononuclear cells that represented macrophages (Fig. 3A). Besides the Th1-attracting CXC chemokines described above, the expression of a fraction of CC chemokines capable of attracting monocytes and macrophages is known to be up-regulated by IFN-γ [17]. Therefore, we examined if the expression of such chemokines might be up-regulated in the GMM-challenges skin. As shown in Fig. 3B, the transcription of CCL2 (MCP-1), CCL4 (MIP-1β), and CCL8 (MCP-2) was induced in the skin challenged with the GMM liposome, but not with empty liposome, in all 3 monkeys analyzed.

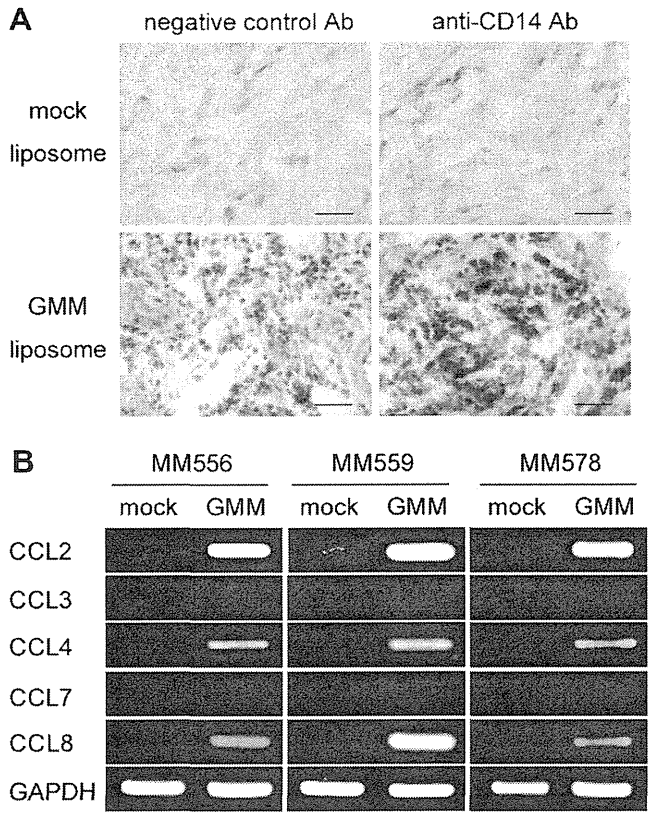

Fig. 3. Up-regulated expression of macrophage-attracting chemokines in the GMM-challenged skin. (A) The GMM-challenged and mock-challenged skin sections derived from MM556 were immunolabeled with either anti-rhesus macaque CD14 antibody or negative control antibody, and viewed under a microscope. Scale bars, 50 μm. (B) Gene expression of indicated CC chemokines was analyzed by RT-PCR, as in Fig. 1.

3.5. An integrated model for the GMM-induced tissue response

As illustrated in Fig. 4, the tissue response to GMM involved up-regulated expression of a set of Th1-attracting chemokines (Fig. 1B) and local accumulation of Th1 T cells that produced IFN-γ (Fig. 2B). This was also associated with up-regulated expression of cytotoxic granule proteins (Fig. 2C) and macrophage-attracting chemokines (Fig. 3B). However, the initial event that triggers these tissue reactions remains to be determined. Although TDM exhibits outstanding adjuvant functions, such activities have not been noted for GMM [5], and indeed, no tissue reactions manifest at the site of GMM injection in unsensitized animals [7], making it unlikely that GMM activates tissue-resident innate immune cells directly. Alternatively, we propose that tissue dendritic cells take up GMM and stimulate GMM-specific T cells localized in their vicinity to release IFN-γ (Fig. 4). Subsequently, tissue-resident macrophages respond to IFN-γ and produce a set of chemokines that are up-regulated by IFN-γ, including those attracting Th1 T cells (CXCL9, CXCL10, and CXCL11) and those attracting macrophages (CCL2, CCL4, and CCL8). The transcriptional expression of CXCR3, the major receptor for CXCL9, CXCL10, and CXCL11 [14], was readily detected in rhesus macaque IFN-γ-producing, GMM-specific T cell lines (D. Morita and M. Sugita, unpublished data), and indeed, circulating GMM-specific T cells were able to extravasate and approach the site of mycobacterial infection where GMM was produced [7]. Therefore, GMM-specific T cells could contribute to the establishment and maintenance of the IFN-γ dominant local milieu, favoring the host to control mycobacterial infections. Similar patterns of chemokine responses are induced in lungs infected

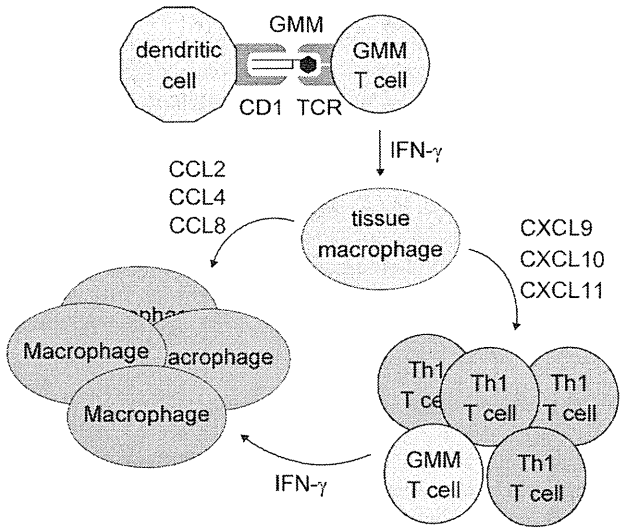

Fig. 4. A proposed model for tissue responses to GMM. IFN-γ produced by activated GMM-specific T cells triggers the up-regulated expression of Th1-attracting chemokines (CXCL9, CXCL10, and CXCL11) and those attracting macrophages (CCL2, CCL4, and CCL8) by tissue-resident macrophages. The local accumulation of IFN-γ-producing T cells, including GMM-specific T cells, further contributes to the establishment of an IFN-γ-dominant niche.

with pathogenic mycobacteria [18], and GMM-specific T cells, including those expressing the high affinity germline-encoded, mycolyl lipid-reactive (GEM) T cell receptor [19], expand prominently in patients with tuberculosis [8]. Taken together, these results suggest that the GMM-elicited tissue response demonstrated in the present study could potentially occur at the site of infection with pathogenic mycobacteria.

The highly potent Th1-inducing peptide, designated Peptide-25, derived from Ag85B has been discovered and studied extensively [20]. The present study indicates that GMM may function as a CD1-presented lipid version of Th1-inducing Ag. Interestingly, the production of GMM depends on mycolyltransferase activities of the Ag85 proteins [5]. Thus, up-regulated expression of Ag85 by proliferating mycobacteria is associated with the creation of potent Th1-inducing peptides (Peptide-25) and lipids (GMM). Given that Ag85 is critical for survival and virulence of mycobacteria, we predict that tissue responses to such Th1-inducing peptides and lipids could function efficiently at the site of infection with pathogenic mycobacteria.

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結核菌の細胞内寄生戦略

——結核菌によるファゴソーム成熟とオートファゴソーム形成阻害

Strategy for intracellular parasitism of *Mycobacterium tuberculosis*

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◎結核菌は細胞内寄生性細菌であり，貪食されたマクロファージ内で増殖することができる．結核菌の細胞内増殖能はファゴリソソーム形成を阻害することによって獲得している．また，結核菌は細胞内寄生性細菌の排除に機能するオートファジー誘導も阻害している．著者らは小胞輸送を制御するRab GTPaseに注目して，ファゴソーム成熟に機能するRab GTPaseを同定した．これらのRab GTPaseが結核菌ファゴソームに局在しないため，ファゴリソソーム形成が阻害されることを明らかにした．つぎに，アクチン結合性蛋白質であるCoronin-1aによる結核菌へのオートファゴソーム形成阻害機構について解析した．Coronin-1aはファゴリソソーム形成を阻害することによって結核菌の細胞内増殖を支持していると考えられていた．Coronin-1aノックダウンマクロファージに感染した結核菌は，オートファゴソーム形成の標的になること，その結果，結核菌の増殖が阻害されることを明らかにした．

Key word　結核菌，マクロファージ，ファゴリソソーム形成，オートファジー

マクロファージは感染初期における強力な生体防御機構である．生体内に侵入した細菌，ウイルス，真菌などの微生物はマクロファージによって貪食される．貪食された微生物は殺菌・分解される．獲得免疫である細胞性免疫においてもマクロファージは機能する．ヘルパーT細胞から産生されるIFN-γによってマクロファージは活性化され，その貪食・殺菌作用が強化される．貪食された微生物はファゴソームで包み込まれる．ファゴソームに初期エンドソームと後期エンドソームが融合することによってファゴソーム成熟が進行し[1]，ファゴソーム成熟過程においてファゴソームにプロトンポンプが局在してファゴソーム内が酸性化される．また，NADPHオキシダーゼが活性化されて，ファゴソーム内で活性酸素が産生される．成熟したファゴソームはリソソームと融合してファゴリソソーム形成を行い，ファゴリソソーム形成によってリソソーム由来の酸性ホスファターゼや蛋白質分解酵素がファゴソーム内に流入する．

この一連の過程によってマクロファージに貪食された微生物は殺菌・分解されるが，細胞内寄生性細菌はさまざまな戦略によって貪食・殺菌作用から回避することができる．

結核菌は細胞内寄生性細菌である

Mycobacterium tuberculosis(結核菌)は細胞内寄生性細菌であり，ヒト肺に感染して肺胞マクロファージに貪食されても殺菌・分解されずにマクロファージ内で増殖することができる[2]．結核菌の細胞内増殖能は，ファゴソーム内の環境を結核菌の増殖に適したニッチへと変化させることによって獲得している．結核菌は，ウレアーゼやスーパーオキシドジムスターゼ，カタラーゼなど

の酵素を産生することによってファゴソーム内の酸性化を阻害したり，活性酸素による殺菌作用から回避している．さらに，ファゴリソソーム形成を阻害することによってリソソームによる殺菌作用からも回避する．結核菌体膜成分の糖脂質はファゴソーム成熟やファゴリソソーム形成を阻害するといわれている．

近年，結核菌もリステリアや赤痢菌と同様にファゴソーム膜を溶解して細胞質へ移行していることが明らかになった[3]．結核菌の近縁種である*Mycobacterium marinum*もリステリアや赤痢菌と同様に，ファゴソーム膜を溶解してファゴソームから細胞質に移行する[4]．さらに，宿主細胞のアクチンを重合させることによって細胞質内を移動することができる．結核菌もファゴソームから細胞質に移行した後にアクチン重合によって細胞質内を移動しているかもしれない．しかし，結核菌や*M. marinum*のファゴソームからの脱出や細胞質内への移行は感染3日後になってようやく起こる．また，結核菌は感染後期において感染マクロファージにネクローシスやアポトーシスを引き起こす．このことは，細胞内寄生性の初期段階の保証はファゴソーム内にとどまってファゴソーム成熟やファゴリソソーム形成を阻害することによって獲得していると考えられる．

結核菌によるファゴソーム成熟阻害機構

マクロファージに感染した結核菌によるファゴリソソーム形成阻害機構として，

① 結核菌がファゴソーム成熟を阻害した結果，ファゴリソソーム形成が阻害される

② アクチン結合性蛋白質であるCoronin-1aが結核菌ファゴソームに局在することによって，リソソームとの融合を阻害する

という2つのモデルが提唱されている．

1. 結核菌がファゴソーム成熟を阻害した結果，ファゴリソソーム形成が阻害される

①に関して，Dereticらの研究グループはファゴソーム成熟に機能するRab GTPaseであるRab5とRab7に注目した．彼らは，結核菌ファゴソームには初期エンドソームマーカーであるRab5が局在した状態であり，後期エンドソームマーカーであるRab7は局在しないことを示している[5]．また，結核菌の細胞壁構成糖脂質が結核菌ファゴソームと初期エンドソームの融合を促進すること，ファゴソーム成熟に必要であるホスファチジルイノシトール三リン酸(PI3P)の合成が結核菌ファゴソームでは抑制されることを示している[6]．さらに，結核菌はPI3PホスファターゼであるSapMを合成・分泌することによって結核菌ファゴソームに局在するPI3Pを分解することができる[7]．以上の結果は，結核菌はファゴソーム成熟を阻害するためにPI3Pを標的にしていることを示す．

また，Rab14とRab22aの結核菌ファゴソームにおける局在とファゴソーム成熟における機能を明らかにした[8,9]．Rab14やRab22aは初期エンドソームに局在するRab GTPaseであるが，ファゴソーム成熟における機能は明らかになっていなかった．Rab14やRab22aはラテックスビーズファゴソームや不活性化した結核菌のファゴソームには局在しないが，結核菌ファゴソームにおいて局在することを明らかにした．不活性化型Rab遺伝子発現やRNA干渉法によるノックダウンによって，結核菌ファゴソームの成熟が進行することを示した．以上の結果は，ファゴソームにRab14やRab22aを局在させることによって結核菌はファゴソーム成熟の進行を阻害することを示唆する．

近年，Rab7が結核菌ファゴソームに局在しないことに関して反対の観察研究が報告されている．Hmamaらの研究グループは結核菌ファゴソームにRab7が局在することを示した[10]．また著者らは，Rab7は一度結核菌ファゴソームに局在して，その後，結核菌ファゴソームから解離することを示している[11,12]．

著者らは，黄色ブドウ球菌ファゴソームと結核菌ファゴソームにおける42のRab GTPaseの局在を網羅的に比較した[12]．Rab GTPaseはRas superfamilyに属するGTPaseであり，60以上のファミリー遺伝子が属している[13]．これまで，エンドサイトーシスやエキソサイトーシスの小胞輸送におけるRab GTPaseの機能は明らかにされてきたが，ファゴソーム成熟やファゴリソソーム形

成におけるRab GTPaseの機能はRab5とRab7以外ほとんど明らかになっていない．そのため，ファゴソーム成熟に関与する新規のRab GTPaseを同定することが期待できた．黄色ブドウ球菌ファゴソームには22のRab GTPaseが局在するが，そのうちの17遺伝子が結核菌ファゴソームから解離する，あるいは局在しないことを明らかにした．

つぎにファゴソーム成熟，とくにファゴソーム内の酸性化とファゴソームへの加水分解酵素であるカテプシンDの輸送に関与するRab GTPaseを，不活性化型Rab遺伝子を発現するマクロファージを用いて調べた．ファゴソーム内の酸性化にはRab7，Rab20，Rab39が関与すること，ファゴソームへのカテプシンDの局在化にはRab7，Rab20，Rab32，Rab34，Rab38が関与することを明らかにした．Rab20は小胞体に局在するRab GTPaseであり，ファゴソーム成熟における機能は明らかになっていなかった．Rab39のリソソーム局在性Rab GTPaseであり，そのファゴソームへの局在は貪食後期にはじまる．このことはRab39は貪食後期におけるファゴソーム内の酸性化に機能していることを示唆する．Rab32，Rab34，Rab38は*trans*-Golgi網(TGN)に局在することが明らかになっている．また，カテプシンDのファゴソームへの輸送は後期エンドソームやリソソームのほかにも，TGNから直接輸送されていることが知られている[14]．

以上の結果は，結核菌はファゴソーム内の酸性化を阻害することによってカテプシンDなどの加水分解酵素をファゴソーム内で活性化させない，さらに後期エンドソーム，リソソーム，TGNからのカテプシンDの結核菌ファゴソームへの輸送を阻害することによってファゴソーム内での結核菌殺菌因子の産生を回避していることを示唆する(図1)．

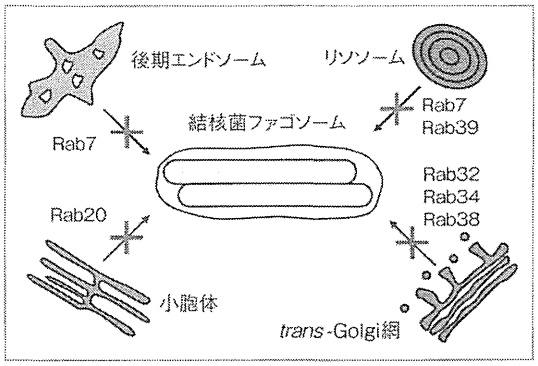

図1　結核菌によるファゴリソソーム形成阻害機構
結核菌ファゴソームにはファゴソーム成熟に関与するRab GTPaseが局在せず，後期エンドソームや小胞体，*trans*-Golgi網との融合が阻害される．そのため，結核菌ファゴソームの酸性化やカテプシンDの輸送が阻害されてファゴソーム成熟が阻害される．その結果，結核菌ファゴソームとリソソームの融合，ファゴリソソーム形成は阻害される．

2. アクチン結合性Coronin-1aは，結核菌オートファゴソーム形成を阻害する

Coronin-1aは，Coroninファミリー遺伝子に属するアクチン結合性蛋白質である．Coroninファミリー遺伝子は，酵母から哺乳類まで多くの真核生物に保存されている[15]．Coroninはアクチンの枝分かれ構造に機能するArp2/3複合体と結合することによって，Arp2/3のアクチン線維からの離脱を制御している．Coronin-1aはヒトおよびマウスではおもに白血球系細胞で発現している．マクロファージにおけるCoronin-1aの機能が明らかになったのは結核菌ファゴソームに局在する蛋白質として同定されたTACO(Tryptophan Aspartate containing COat protein)によってである．

②に関しては，Pietersらの研究グループによって解析されてきた．Pietersらは結核ワクチン株であるBCGをマクロファージに感染させて生化学的にBCGファゴソームを単離した[16]．そして，BCGファゴソームに特異的に局在する蛋白質を探索した結果，TACO，すなわちCoronin-1aを同定した．Coronin-1aは死菌BCGファゴソームには一時的にしか局在しないが，生菌BCGファゴソームには継続的に局在していることを明らかにした．つぎに，Coronin-1aノックアウトマウスを作製して，そのノックアウトマウス由来マクロファージに結核菌を感染させた[17]．Coronin-1aノックアウトマウス由来マクロファージにおいて，結核菌ファゴソームのファゴリソソーム形成が行われて結核菌の増殖が抑制されることを明らかにした．また，カルシニューリンがCoronin-

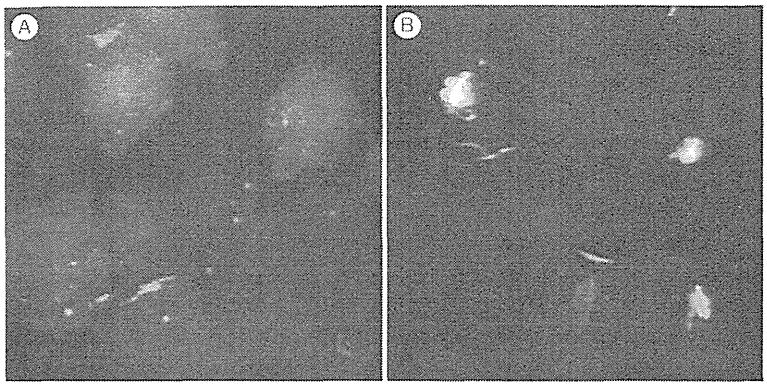

図 2　Coronin-1aノックダウンマクロファージにおいて感染結核菌にLC3が局在化する
マクロファージに感染した結核菌へのオートファジーマーカー蛋白質であるLC3の局在を示している．GFP融合LC3蛋白質を恒常的に発現するマクロファージに結核菌を感染させた．コントロールマクロファージ(A)ではLC3は結核菌に局在しなかったが，Coronin-1aノックダウンマクロファージ(B)では結核菌にLC3が局在した．

1aによって活性化されることを見出し，FK506やシクロスポリンなどのカルシニューリン阻害剤は結核菌ファゴソームのファゴリソソーム形成を促進することを明らかにした．しかし，Coronin-1aの結核菌ファゴソームへの持続的な局在機構やカルシニューリンによるファゴリソソーム形成阻害機構について，その詳細は明らかになっていない．

オートファジーは飢餓などで誘導される蛋白質分解機構であり，細胞や生体の恒常性の維持に機能する[18]．近年，オートファジーは細胞内寄生性細菌の排除にも関与していることが明らかになっている．結核菌感染マクロファージにおいても飢餓，ラパマイシン，IFN-γによって誘導したオートファジーによって結核菌を排除することができる[19]．しかし，定常状態の結核菌感染マクロファージではオートファジーは誘導されない．

著者らは結核菌感染マクロファージにおいてCoronin-1aがオートファゴソーム形成を阻害して，その結果，結核菌の細胞内増殖を支持していることを見出した[20]．すなわち，Coronin-1aノックダウンマクロファージにおいて結核菌の細胞内増殖は抑制されるが，同時にオートファジー関連遺伝子もノックダウンすると結核菌増殖は回復することを明らかにした．さらに，結核菌ファゴソームにオートファジーマーカーであるLC3が局在して結核菌オートファゴソームが形成されること(図2)，LC3陽性結核菌ファゴソームにオートファジーアダプター蛋白質であるp62/SQSTM1が局在して結核菌ファゴソームのポリユビキチン化が促進されることを明らかにした．また，Coronin-1aノックダウンマクロファージでは結核菌感染によってp38MAP kinaseが特異的に活性化した．以上の結果は，結核菌感染によって活性化されるオートファジーを誘導するシグナル伝達経路がCoronin-1aによって阻害されている可能性を示唆する．

おわりに

結核菌はマクロファージに貪食されても結核菌ファゴソームへのファゴソーム成熟に関与するRab GTPaseの局在をさせないことによってファゴソームの酸性化と加水分解酵素の局在化を阻害している．また，結核菌ファゴソームに局在する宿主因子であるアクチン結合性蛋白質であるCoronin-1aはオートファゴソーム形成を阻害する因子であり，感染結核菌のオートファゴソーム形成を阻害している．結核菌はファゴリソソーム形成やオートファゴソーム形成による殺菌・分解機構から回避することによって細胞内増殖を行う戦略を採用している．

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Immunogenicity of dormancy-related antigens in individuals infected with *Mycobacterium tuberculosis* in Japan

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SUMMARY

SETTING: DosR regulon genes are considered essential for *Mycobacterium tuberculosis* dormancy, and their products are demonstrated to have immunogenicity in *M. tuberculosis*-infected individuals, suggesting that DosR regulon-encoded proteins are suitable targets for vaccines to control the reactivation of dormant *M. tuberculosis*.

OBJECTIVE: Prospective analysis of T-cell and antibody responses against DosR regulon-encoded antigens in *M. tuberculosis*-infected individuals in Japan to identify effective vaccine targets.

DESIGN: T-cell responses against 33 DosR regulon-encoded antigens were investigated in 26 consecutive *M. tuberculosis*-infected individuals—14 with latent tuberculosis infection (LTBI) and 12 with active pulmonary tuberculosis (PTB)—using enzyme-linked immunosorbent spot assay, and antibody responses in 42 consecutive individuals, 14 with LTBI and 28 with PTB.

RESULT: Six antigens (Rv0570, Rv1996, Rv2004c, Rv2028c, Rv2029c and Rv3133c) induced stronger T-cell responses in LTBI than in PTB, In contrast, antigen-specific antibody responses to five antigens (Rv0080, Rv1738, Rv2007c, Rv2031c and Rv2032) were found to be stronger in PTB than in LTBI cases.

CONCLUSION: T-cell responses to six antigens might contribute to natural protection against dormant *M. tuberculosis*. These antigens are therefore considered to be potential targets of novel vaccines to control *M. tuberculosis* reactivation in the Japanese population.

KEY WORDS: *Mycobacterium tuberculosis*; vaccine; dormancy; DosR regulon

TUBERCULOSIS (TB), an epidemic disease caused by *Mycobacterium tuberculosis,* remains a serious global human health problem. In 2010, TB incidence and mortality were respectively 8.8 and 1.4 million.[1] Approximately 5–10% of people infected with *M. tuberculosis* develop active TB at some time during their life, while in the remaining individuals *M. tuberculosis* infection remains latent, without clinical symptoms.[2–4]

In individuals with latent TB infection (LTBI), *M. tuberculosis* bacilli persist in the human body, particularly in the lung granuloma, and can survive for long periods of time in a reversible, metabolically inactive state. Some LTBI develop into active TB due to reactivation of *M. tuberculosis* if the host cell-mediated immune response, which plays a pivotal role in the protection against TB, is impaired by a causative agent such as aging, human immunodeficiency virus (HIV) infection or immunosuppressive treatment. This reactivation of persisting *M. tuberculosis* bacilli is the major cause of adult pulmonary TB.[2–4]

M. bovis bacille Calmette-Guérin (BCG) is currently the only available prophylactic vaccine against *M. tuberculosis*. BCG vaccination of newborns and infants is an effective preventive measure against initial active TB disease; however, its prophylactic efficacy in adults is limited.[5–7] Various anti-tuberculosis vaccine forms such as DNA vaccines, recombinant BCG vaccines, subunit vaccines and attenuated TB vaccines are under development, but none has so far achieved an effect superior to BCG.[5–7] As adult TB is largely due to the reactivation of dormant *M. tuberculosis*, the development of more effective vaccines, especially to prevent reactivation, is urgently needed.

The 48 genes of the DosR regulon, some of which are considered essential for *M. tuberculosis* dormancy, are upregulated by stresses such as hypoxia, low pH, nitric oxide and carbon monoxide.[8–10] Such evidence

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suggests that DosR regulon plays an essential role in adaptation to these stresses, although the detailed functions of most proteins encoded in this regulon are not fully understood. These DosR regulon-encoded proteins have also been demonstrated to have immunogenicity in *M. tuberculosis*-infected individuals,[11–16] suggesting that they are suitable targets for vaccines to control the reactivation of dormant *M. tuberculosis*.

Immune responses against *M. tuberculosis* have been preferentially measured by interferon-gamma (IFN-γ) release assays (IGRA), as the IFN-γ produced by $CD4^+$ helper T-cells (Th1 cells) and $CD8^+$ cytotoxic T-cells plays a central role in the protection against *M. tuberculosis*.[17–19] IGRA was used to evaluate T-cell responses against DosR regulon-encoded antigens in blood samples mainly from European (low-burden) and African (high-burden) countries, but the immune response profiles were somewhat different.[11–16] The difference may stem from ethnic differences, bacterial prevalence, BCG vaccination history or detection methods, as well as genetic differences in local pathogens.

In this prospective study, we comparatively analysed T-cell and antibody responses against 33 DosR regulon-encoded antigens in consecutive individuals with LTBI and active pulmonary TB (PTB) in Japan, an Asian country with an intermediate TB burden, to identify effective vaccine targets.

MATERIALS AND METHODS

Study subjects

This prospective study was approved by the institutional review board of the Hamamatsu University School of Medicine, Hamamatsu, Japan, and conducted according to the principles expressed in the Declaration of Helsinki. Only adults (aged ≥20 years) who provided written informed consent were included in the study. Twenty-six consecutive Japanese individuals, 14 with LTBI and 12 patients with active PTB, were recruited to investigate T-cell responses against DosR regulon-encoded antigens (Table 1). Eleven healthy controls were recruited to check T-cell responses against 6 kDa early secretory antigenic target (ESAT-6) and 10 kDa culture filtrate protein (CFP-10), in addition to the LTBI and PTB patients. For serum antibody responses against DosR regulon-encoded antigens, another 16 PTB patients were also examined (Table 2). A total of 42 consecutive individuals, 14 with LTBI and 28 with PTB, were recruited for antibody responses.

All LTBI cases were asymptomatic volunteers or non-TB patients positive on the QuantiFERON®-TB Gold In-Tube (QFT) test (Cellestis, VIC, Australia). None had any evidence of active TB on clinical examination, chest radiography (CXR) and microbiological examinations of sputum and/or bronchial lavage.

Table 1 Characteristics of study participants for T-cell responses (ELISpot)

	LTBI* (n = 14) n (%)	PTB† (n = 12) n (%)	Total (N = 26) n (%)
Age, years, median [range]	71 [33–84]	78 [33–97]	75 [33–97]
Male sex	10 (71.4)	2 (16.7)	12 (46)
Recent exposure‡	3 (21.4)	0	3 (11.5)
BCG vaccination§	11 (78.6)	9 (75.0)	20 (76.9)

*Individuals with LTBI who were QFT-positive but had no clinical evidence of active TB.
†Patients with active PTB confirmed by microbiological examination.
‡History of contact with an active TB patient in the last 6 months.
§Vaccination with *Mycobacterium bovis* BCG.
ELISpot = enzyme-linked immunosorbent spot; LTBI = latent tuberculous infection; PTB = pulmonary tuberculosis; BCG = bacille Calmette-Guérin; QFT = QuantiFERON®-TB Gold In-Tube.

PTB was thus excluded among the LTBI patients, and no LTBI patient had developed PTB at the time of writing. The diagnosis of PTB by expert physicians was confirmed using Ziehl-Neelsen staining of sputum smears and bacterial culture for *M. tuberculosis*.

Individuals with infectious diseases other than TB, immunosuppressive therapy, chemotherapy, autoimmune or haematological diseases or HIV infection were excluded from the study. All healthy controls were QFT-negative and had no history of close TB exposure, anti-tuberculosis treatment or prior PTB. Their CXRs were normal, and they had no respiratory or systemic symptoms. All of the healthy controls had been BCG vaccinated.

Preparation of recombinant proteins

DNA encoding DosR regulon proteins were amplified by polymerase chain reaction (PCR) using genomic DNA from *M. tuberculosis* H37Rv or BCG as template. Primers were designed according to the genetic information obtained from the TubercuList (http://genolist.pasteur.fr/TubercuList/). Appropriate restriction sites were introduced at the 5′ and 3′ ends of the DNA by PCR, and the PCR products were digested and ligated into the corresponding restriction sites of pET-28b(+) vectors (Novagen/Merck, Darmstadt,

Table 2 Characteristics of study participants for antibody titration (ELISA)

	LTBI* (n = 14) n (%)	PTB† (n = 28) n (%)	Total (N = 42) n (%)
Age, years, median [range]	71 [33–84]	81.5 [33–97]	73 [33–97]
Male sex	10 (71.4)	14 (50.0)	24 (57.1)
Recent exposure‡	3 (21.4)	0	3 (7.1)
BCG vaccination§	11 (78.6)	17 (60.7)	28 (66.7)

*Individuals with LTBI who were QFT-positive but had no clinical evidence of active TB.
†Patients with active PTB confirmed by microbiological examination.
‡History of contact with an active TB patient in the last 6 months.
§Vaccination with *Mycobacterium bovis* BCG.
ELISA = enzyme-linked immunosorbent assay; LTBI = latent tuberculous infection; PTB = pulmonary tuberculosis; BCG = bacille Calmette-Guérin; QFT = QuantiFERON®-TB Gold In-Tube.

Germany). Plasmids were purified using Plasmid Purification Kits (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions. The nucleotide sequences were confirmed by automated DNA sequencing (ABI PRISM 310 Genetic Analyzer; Applied Biosystems, Foster City, CA, USA) using a dye primer cycle sequencing kit (Applied Biosystems).

Escherichia coli BL21(DE3) competent cells (Novagen/Merck) were transformed with pET-28(+) vectors containing DNA for the DosR regulon proteins or pET-23(−) vectors containing those for ESAT-6 or CFP-10. Proteins were induced with isopropyl β-D(−)-thiogalactopyranoside (IPTG; Wako Pure Chemical Industries, Osaka, Japan). Proteins were extracted from the bacterial cells with 8M urea (Wako Pure Chemical Industries) and purified by Ni^{2+}-nitrilotriacetic acid (Ni^{2+}-NTA) agarose (Qiagen), according to the manufacturer's instructions. Some proteins were further purified by gel filtration using Superdex 200 HR 10/30 (GE Healthcare, Chalfont St. Giles, UK). The purity and size of recombinant proteins were checked using sodium dodecyl sulfate polyacrylamide gel electrophoresis. Residual endotoxin levels were determined by Endospecy ES-24S Kit and Toxicolor DIA Kit (Seikagaku Biobusiness Corporation, Tokyo, Japan), and were found to be <50 international units/mg of recombinant protein.

Enzyme-linked immunosorbent spot assay

In PTB patients, blood samples were obtained within 2 weeks of the initiation of treatment. Peripheral blood mononuclear cells (PBMCs) were isolated from fresh venous blood by Ficoll-Paque PREMIUM (GE Healthcare). ELISpot assay (Millipore House, Watford, UK) was performed with an antibody pair, horseradish peroxidase (HRP) conjugated streptavidin, and 3-amino-9-ethylcarbazole (AEC) chromogen (Human IFN-γ ELISpot pair, Streptavidin-HRP ELISpot, and AEC Substrate Set, respectively; BD Biosciences, Franklin Lakes, NJ, USA), according to the manufacturer's instructions. PBMCs (2×10^5) suspended in 200 μl RPMI-1640 medium (Wako Pure Chemical Industries) containing 10% human AB serum (Sigma-Aldrich, St. Louis, MO, USA) were stimulated in duplicate for 18 h with 1 μg recombinant protein (final concentration 5 μg/ml) in MultiScreen 96-well plates (Millipore, Billerica, MA, USA) precoated with anti-human IFN-γ capture antibody. Purified protein derivative (PPD; final concentration 5 μg/ml; Japan BCG Laboratory, Tokyo, Japan) and phytohaemagglutinin (PHA; final concentration 5 μg/ml; Sigma-Aldrich) were also used as stimulators. The number of spot-forming cells (SFC) was manually counted under a dissecting microscope by two operators blinded to the clinical information. The results are expressed as SFC count/10^6 cells after subtracting the mean SFC count in the medium control (<20 SFC/10^6 PBMCs in all subjects). Assays were considered valid if there were at least 250 SFC/10^6 PBMCs against PHA. Discordant SFC results were resolved by consensus between both operators.

Enzyme-linked immunosorbent assay

The enzyme-linked immunosorbent assay (ELISA) was performed as described elsewhere[20] using 96-well flat-bottomed half-well plates (Corning Inc, Corning, NY, USA) pre-coated with DosR regulon-encoded protein (100 μg/well). After blocking with Block One Blocking solution (Nacalai Tesque, Kyoto, Japan), 100 μl of diluted serum (1:100) with phosphate buffered saline containing 5% Block One Blocking solution and 0.05% Tween-20 (Sigma-Aldrich) was added to each well in duplicate. HRP-conjugated goat anti-human immunoglobulin G (IgG; Invitrogen, Carlsbad, CA, USA) was used as a secondary antibody, and colour development was performed using TMB One Component HRP Microwell Substrate (BioFX Laboratories, Owings Mills, MD, USA). Optical density was measured with an ELISA reader (EZS-ABS; IWAKI, Tokyo, Japan), and the result of the negative control was subtracted from each experimental result.

Data analysis

Antigen-specific T-cell and serum antibody responses were compared between the LTBI and PTB groups using the Mann-Whitney *U* test for non-parametric comparison. In all analyses, $P < 0.05$ was regarded as significant. All data were analysed using commercially available software (JMP version 9.0.3; Statistical Analysis Software Institute, Cary, NC, USA).

RESULTS

Establishment of ELISpot assay system for antigen-specific T-cell response

We first established an ELISpot assay and validated the specificity and sensitivity using PBMCs from study subjects (Table 1). Stimulation with PHA induced > 1000 SFC/10^6 PBMCs in all subjects (data not shown), indicating no obvious immunosuppression in study participants. As shown in Appendix Table A.1 and Appendix Figure A.1, T-cell responses against ESAT-6 and CFP-10 in LTBI and PTB patients were significantly stronger than those in healthy controls.* As SFC against both antigens in healthy controls were <30/10^6 PBMCs, the newly established ELISpot assay seemed comparable to the T-SPOT®.*TB* assay (Oxford Immunotec, Abingdon, UK), defining >32 SFC/10^6 PBMCs to these antigens as positive. In addition, when this cut-off value was applied to the newly established ELISpot assay system, the results

*The Appendix is available in the online version of this article at http://www.ingentaconnect.com/content/iuatld/ijtld/2012/00000017/00000006/art00021

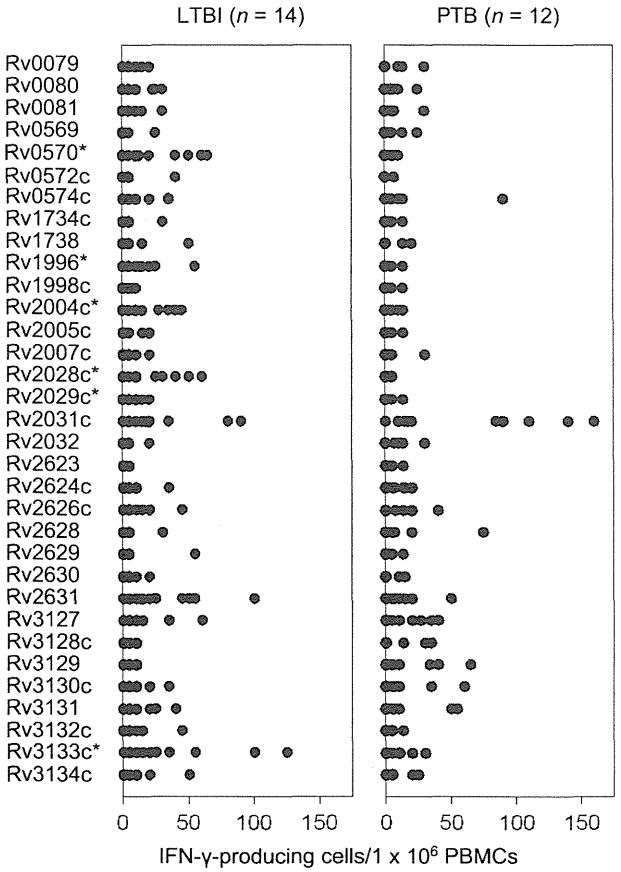

Figure 1 T-cell responses to individual 33 DosR regulon-encoded antigens. The number of IFN-γ-producing cells in response to 33 DosR regulon-encoded antigens was measured by ELISpot assay. *T-cell responses that were significantly stronger in LTBI than in PTB cases. Analyses were conducted using the Mann-Whitney *U* test for non-parametric comparison; $P < 0.05$ was regarded as significant. LTBI = latent tuberculous infection; PTB = pulmonary tuberculosis (see Table 1 for details); IFN-γ = interferon-gamma; PBMC = peripheral blood mononuclear cells.

were consistent with those of the QFT test conducted before this study.

T-cell responses against DosR regulon-encoded proteins

We next examined T-cell responses against DosR regulon-encoded proteins using the ELISpot assay comparing LTBI and PTB cases. As we could not prepare some of the recombinant DosR proteins in the bacterial expression system employed, we analysed the immunogenicity of 33 DosR proteins. As shown in Appendix Table A.1 and Figure 1, T-cell responses against DosR regulon-encoded proteins in LTBI cases seemed stronger than those in PTB patients. When the results were analysed statistically, T-cell responses against six DosR regulon-encoded proteins (Rv570, Rv1996c, Rv2004, Rv2028, Rv2029c and Rv3133c) were significantly stronger in LTBI than in PTB patients (Figure 2). Although some materials from LTBI and PTB patients showed strong T-cell responses against

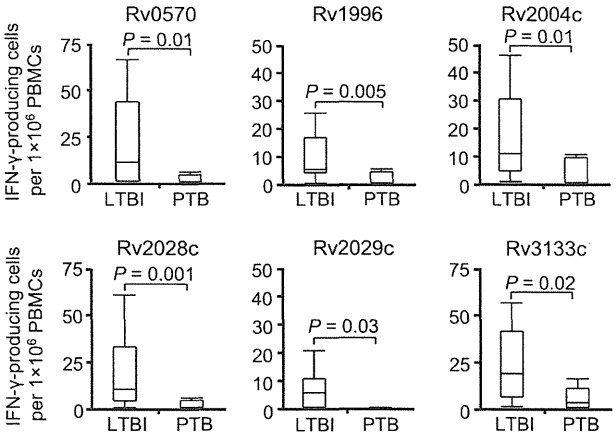

Figure 2 T-cell responses to DosR regulon-encoded antigens with stronger immunogenicity in individuals with LTBI than in PTB. The number of IFN-γ-producing cells in response to 33 DosR regulon-encoded antigens was measured by ELISpot assay. The results are shown in box-and-whisker plots. The line within the box shows the median SFC value. Lower and upper boundaries of the box indicate the 25th and 75th percentiles. The whiskers represent the lowest and highest data within 1.5 interquartile ranges of the lower and upper quartiles, respectively. T-cell responses significantly higher in LTBI than in PTB patients are shown. IFN-γ = interferon-gamma; PBMC = peripheral blood mononuclear cells; LTBI = latent tuberculous infection; PTB = pulmonary tuberculosis; SFC = spot-forming cells.

Rv2031c (Figure 1 and Appendix Table A.1), no statistical difference was observed between the groups.

Serum antibody responses against DosR regulon-encoded proteins

Antibody responses against DosR regulon-encoded antigens were also examined using ELISA in 16 TB patients other than those included in the ELISpot analysis (Table 2). The results were different from those for T-cell responses, showing no stronger antigen-specific antibody response in LTBI than in PTB (Appendix Table A.2; Figure 3). In contrast, antibody responses to the five antigens (Rv0080, Rv1738, Rv2007c, Rv2031c and Rv2032) were stronger in PTB than in LTBI patients (Figure 4).

DISCUSSION

DosR regulon-encoded proteins are considered good targets for new vaccines to prevent the reactivation of *M. tuberculosis* due to their unique expression profiles and functions in dormant *M. tuberculosis*;[8–10] immune responses to these proteins have been studied in humans[11–16] and mice.[21,22] In the present study, we found that human T-cell responses to at least six DosR regulon-encoded proteins were stronger in LTBI than in PTB patients in a Japanese population. Leyten et al. showed that cumulative T-cell responses to DosR regulon-encoded antigens were stronger in healthy tuberculin skin test (TST) positive individuals than in PTB patients.[11] T-cell responses to PPD in

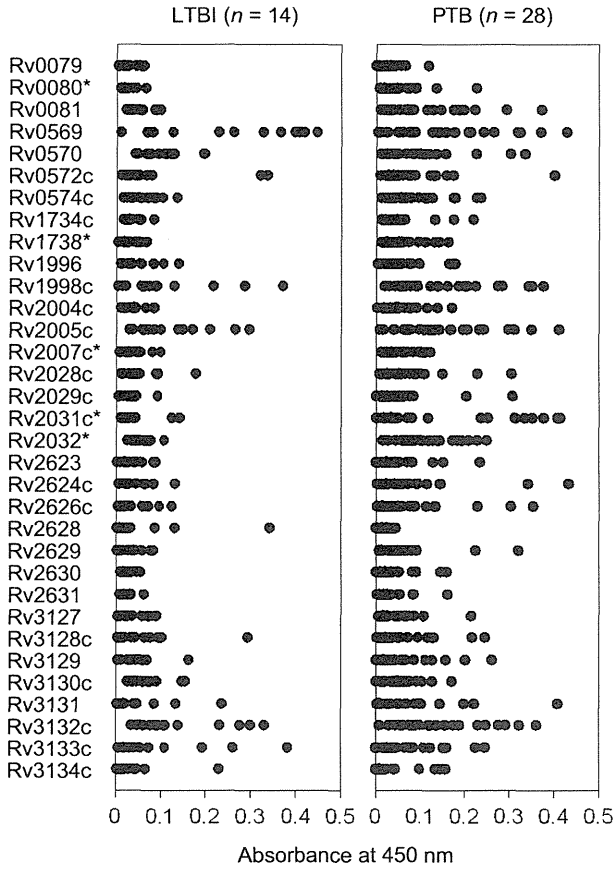

Figure 3 Serum antibody responses against 33 DosR regulon-encoded antigens. Serum IgG responses were measured by ELISA using 1:100 diluted serum and HRP-labelled polyclonal anti-human IgG antibodies. *Serum IgG responses that were significantly stronger in PTB than in LTBI. LTBI = latent tuberculous infection; PTB = pulmonary tuberculosis (see Table 2 for details); IgG = immunoglobulin G; ELISA = enzyme-linked immuno-sorbent assay; HRP = horseradish peroxidase.

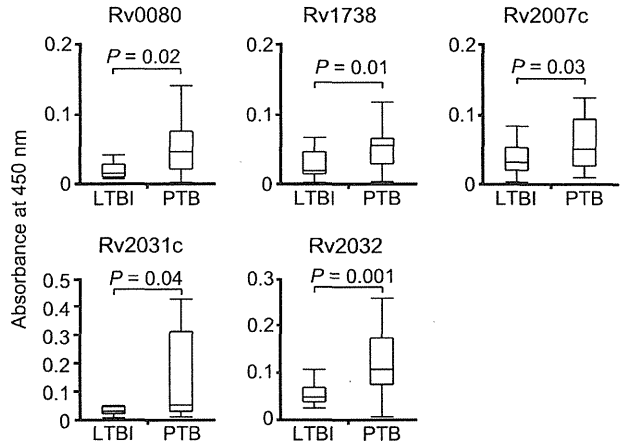

Figure 4 Serum antibody responses against DosR regulon-encoded antigens with stronger immunogenicity in PTB than in LTBI. Serum IgG responses were measured by ELISA using 1:100 diluted serum and HRP-labelled polyclonal anti-human IgG antibodies. IgG responses that were significantly higher in PTB than in LTBI are shown. LTBI = latent tuberculous infection; PTB = pulmonary tuberculosis; IgG = immunoglobulin G; ELISA = enzyme-linked immunosorbent assay; HRP = horseradish peroxidase.

LTBI patients were also reported to be stronger than in PTB patients.[23,24] Although the populations of TST-positive and QFT-positive individuals are not necessarily the same, these observations together imply that immune responses to DosR regulon-encoded antigens, as well as whole TB antigens, might contribute to natural protection against dormant *M. tuberculosis* and that the lack of or decrease in the responses might be associated with the development of PTB. The six DosR regulon-encoded proteins effectively inducing T-cell responses in LTBI are therefore considered to be potential candidate antigens to prevent the reactivation of *M. tuberculosis*, although vaccination effects should be confirmed by animal experiments.

Human T-cell responses to individual DosR regulon-encoded proteins in LTBI and/or PTB have also been reported by some studies.[11,13,16] Leyten et al. showed strong Rv1733c-, Rv2029c-, Rv2627c- and Rv2628-induced IFN-γ responses in TST-positive individuals.[11] Black et al. showed that four DosR regulon-encoded proteins (Rv0080, Rv1733c, Rv1735c and Rv1737c) were recognised most frequently by T-cells in TST-positive and/or ESAT-6/CFP-10-positive individuals in three high-burden African countries.[13] Goletti et al. reported that IFN-γ response to Rv2628 might distinguish recent from remote infection.[16] However, the results, including those of the present study, are somewhat varied; this might stem from differences in ethnic background, bacterial prevalence, BCG vaccination history and detection methods, as well as genetic differences in local pathogens.

Human T-cell response to an antigen is restricted by the human leukocyte antigen (HLA); the distribution profiles of HLA alleles differ by ethnic group. Differences in genetic backgrounds (European vs. African vs. Asian) may thus be a cause of the difference in immunogenicity. Bacterial prevalence may also affect T-cell responses to DosR antigens. Rv2031c (also termed HspX or α-crystallin) was reported to be more strongly recognised by T-cells from LTBI and/or PTB patients than by those from healthy controls.[11,24–26] T-cell responses to Rv2031c in PTB patients were stronger than in LTBI individuals in a low-burden country (the Netherlands), whereas the reverse was true for high-burden countries (African countries).[25] In the present study, although some strong responders to Rv2031c were preferentially found in PTB patients, there was no statistical difference in T-cell responses to Rv2031c as a whole between PTB and LTBI cases in Japan, an intermediate-burden country. Rv2031c was reported to be required for the maintenance of long-term viability during latent infection and replication during initial *M. tuberculosis* infection.[27] Together with reports demonstrating that

vaccination with Rv2031c induced protective immunity in animal studies, Rv2031 seems to be one of the better target antigens for a novel vaccine(s),[28,29] although racial and/or environmental factors may affect the immune response. BCG vaccination also seems to affect the results, as DosR regulon genes are highly conserved between *M. tuberculosis* and BCG.[14] However, previous reports have shown that BCG vaccination did not induce immune responses to DosR regulon-encoded antigens,[12,23,26] and we also have similar results (Appendix Figure A.2). In addition to the factors described above, differences in detection methods might affect the results. Previous studies mainly employed IFN-γ ELISA with long-term culture (approximately 1 week), which is more sensitive than short-term cultured assay,[30] and the results reflect the entire amount of IFN-γ produced in the culture supernatant, but not the actual number of antigen-specific T-cells. In the present study, we used short-term ELI-Spot assay, which reflects the number of antigen-specific T-cells and is more sensitive than other assays for low-level responses.[31,32] Multi-colour flow cytometric analysis may be the best way of investigating the status of antigen-specific T-cells such as memory/effector, cytokine profiles and gene expression.[33] However, ex vivo multi-colour flow cytometric analysis requires a large amount of blood, as the number of T-cells specific for DosR regulon-encoded antigen is low.[15]

We also analysed serum IgG responses against DosR regulon-encoded antigens. However, the results were not consistent with those of the ELISpot assay. In contrast to T-cell responses, antibody responses to five antigens were found to be stronger in PTB than in LTBI cases. We and others reported similar discordance between T-cell and antibody immune responses to DosR-encoded antigens in DNA-vaccinated mice,[21,22] but this may reflect the immunogenicity of individual antigens. In humans, accumulating evidence suggests that many antibody responses reflect bacillary burden.[34,35] Therefore, the five antigens that invoked stronger antibody responses in PTB than in LTBI cases might also be produced in PTB, and the antibody responses to those might reflect the bacterial number in the body. If so, antibody responses to these antigens might be applicable for a biomarker to distinguish these groups, although a larger-scale study is necessary.

In conclusion, six DosR regulon-encoded proteins were found to induce stronger T-cell responses in LTBI than in PTB cases, and are considered potential candidate antigens for establishing novel vaccines to control *M. tuberculosis* reactivation in Japan.

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APPENDIX

Table A.1 T-cell responses to DosR regulon-encoded antigens

Antigen name	Gene	IFN-γ-producing cells/1 × 10^6 PBMCs, median (range) LTBI*	PTB*	*P* value†
Rv0079		5 (0–20)	0 (0–30)	0.08
Rv0080		5 (0–30)	2.5 (0–25)	0.31
Rv0081		5 (0–30)	0 (0–30)	0.20
Rv0569		0 (0–25)	0 (0–25)	0.28
Rv0570	*nrdZ*	10 (0–65)	0 (0–5)	0.01‡
Rv0572c		0 (0–40)	0 (0–5)	0.10
Rv0574c		7.5 (0–35)	0 (0–90)	0.18
Rv1734c		0 (0–30)	0 (0–10)	0.61
Rv1738		2.5 (0–50)	0 (0–20)	0.15
Rv1996		5 (0–55)	0 (0–10)	0.005‡
Rv1998c		0 (0–10)	0 (0–10)	0.51
Rv2004c		3.75 (0–45)	0 (0–10)	0.01‡
Rv2005c		0 (0–20)	0 (0–10)	0.38
Rv2007c	*fdx*A	0 (0–20)	0 (0–30)	0.59
Rv2028c		10 (0–60)	0 (0–5)	0.001‡
Rv2029c	*pfk*B	5 (0–20)	0 (0–10)	0.03‡
Rv2031c	*Acr*	16.3 (0–90)	51.3 (0–140)	0.15
Rv2032	*Acg*	2.5 (0–20)	0 (0–30)	0.60
Rv2623	*TB31.7*	0 (0–5)	0 (0–10)	0.45
Rv2624c		2.5 (0–35)	2.5 (0–20)	0.80
Rv2626c		5 (0–45)	0 (0–40)	0.55
Rv2628		0 (0–30)	0 (0–75)	0.72
Rv2629		0 (0–55)	0 (0–10)	0.59
Rv2630		0 (0–20)	0 (0–15)	0.18
Rv2631		15 (0–100)	7.5 (0–50)	0.11
Rv3127		10 (0–60)	2.5 (0–40)	0.30
Rv3128c		0 (0–10)	0 (0–35)	0.90
Rv3129		5 (0–10)	2.5 (0–65)	0.98
Rv3130c		5 (0–35)	0 (0–60)	0.19
Rv3131		5 (0–40)	0 (0–55)	0.34
Rv3132c	*devS*	2.5 (0–45)	0 (0–10)	0.31
Rv3133c	*dosR*	17.5 (0–125)	2.5 (0–30)	0.02‡
Rv3134c		0 (0–50)	2.5 (0–25)	0.98
Rv3875 (ESAT-6)	*esxA*	100 (20–280)	90 (15–620)	0.66
Rv3874 (CFP-10)	*esxB*	42.5 (10–505)	45 (20–160)	0.92

*See Table 1.
†T-cell responses (IFN-γ-producing cells) were compared between the LTBI and PTB groups using the Mann-Whitney *U* test for non-parametric comparison.
‡Statistically significant, $P < 0.05$.
IFN-γ = interferon-gamma; PBMC = peripheral blood mononuclear cells; LTBI = latent tuberculous infection; PTB = pulmonary tuberculosis; ESAT-6 = 6 kDa early secreted antigenic target 6; CFP = 10 kDa culture filtrate protein.

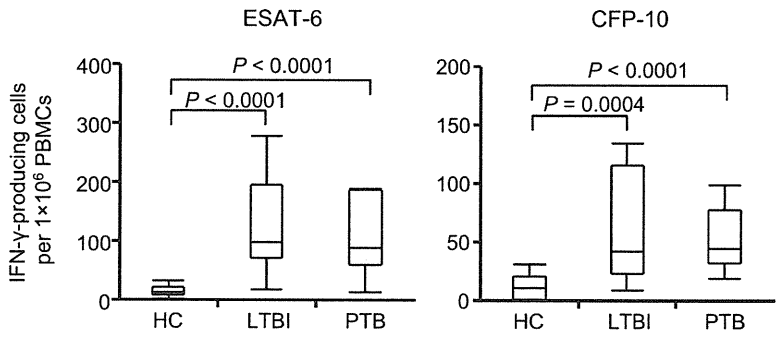

Figure A.1 T-cell responses to ESAT-6 and CFP-10. The number of IFN-γ-producing cells in response to the antigens was measured by ELISpot assay, and the results are shown by box-and-whisker plots. The line within the box shows the median value of spot-forming cells. The lower and upper boundaries of the box indicate respectively, the 25th and 75th percentile. The whiskers represent the lowest and highest data still within 1.5 interquartile ranges of the lower and upper quartiles, respectively. In addition to individuals with LTBI and PTB described in the Materials and Methods, 11 healthy controls were recruited. Antigen-specific T-cell responses were compared between three groups (HC, LTBI and PTB) using Steel-Dwass test for non-parametric multiple comparison. In all analyses, $P < 0.05$ was regarded as significant. ESAT-6 = 6 kDa early secretory antigenic target of *Mycobacterium tuberculosis*; CFP-10 = 10 kDa culture filtrate protein; IFN-γ = interferon-gamma; PBMC = peripheral blood mononuclear cells; HC = healthy controls; LTBI = latent tuberculous infection; PTB = pulmonary tuberculosis.