統計処理　統計処理はStudent's t testを用いて行った。

倫理面への配慮　本研究においてはヒトに由来する試料を用いない。動物実験においては、動物愛護の精神に則り、研究機関の承認を得た上でその規則を遵守して行った。

C. 研究結果

GMM 接種によって誘起される皮膚応答の肉眼的観察　BCG に感染したモルモットにおいて、PPD 接種によりおおよそ 48 時間をピークとした皮膚の硬結を認め、典型的な遅延型アレルギー応答が誘起された。この応答は、BCG 非感染個体ではまったく認めなかったことから、BCG 感作が適切に成立していることが確認された。また、BCG 感作個体においても非感作個体においても、空リポソームの皮内接種部位には皮膚応答を認めなかったことから、リポソーム成分による非特異的応答は無視できると判断した。一方、BCG 感作個体に GMM リポソームを接種したところ、PPD に対する応答よりは弱いものの、おおよそ 48 時間をピークとした皮膚の硬結を観察した。この応答は BCG 非感作個体では認めなかった。

BCG 接種により誘起される皮膚応答の組織学的所見　BCG 感作個体において、PPD 接種部位では顕著な単核球浸潤を認め、CT7 陽性ヘルパー／インデューサーT 細胞および CT6 陽性キラーT 細胞が多数浸潤していた。空リポソーム接種部位には顕著な組織所見の変化を認めなかったのに対し、BCG 感作個体の GMM リポソーム接種部位において単核球の局所浸潤を認めた。浸潤細胞には、CT7 陽性ヘルパー／インデューサーT 細胞および CT6 陽性キラーT 細胞が含まれていた。一方、非感作個体では著変を認めなかった。以上の観察から、GMM に対する皮膚応答は、1) 感作を必要とすること、2) 48 時間をピークとすること、3) 単核球の浸潤を主体とすること、の 3 点から、遅延型アレルギー応答（4 型アレルギー）の定義を満たすものである。従って、糖脂質が遅延型アレルギー応答の誘起アレルゲンとなることが本研究により初めて示された。

所属リンパ節におけるサイトカイン応答　BCG 感作個体より鼠径リンパ節を単離し、リンパ節細胞を GMM リポソーム存在下あるいは非存在下で培養したところ、GMM 刺激特異的な IFN-γの遺伝子発現誘導を観察した。この応答は抗モルモット CD1 抗体により完全に阻害されたのに対し、アイソタイプの一致したネガティブコントロール抗体ではまったく阻害を受けなかったことがら、CD1 依存的応答であることが示された。

TH1 型サイトカイン（IFN-γ、TNF-α）および TH2 型サイトカイン（IL-5、IL-10）について検討したところ、リンパ節細胞は GMM 刺激により IFN-γに加えて TNF-αの遺伝子発現が亢進したが、TH2 型サイトカインの発現誘導をまったく認めなかった。一方、同様の方法でリンパ節細胞を PPD で刺激したところ、TH1 型サイトカインおよび TH2 型サイトカイン両方の遺伝子発現を観察した。以上の結果から、GMM を標的と下 CD1 依存的 T 細胞反応は、極めて TH1 型サイトカイン応答にシフトした応答であることが明らかとなった。

D. 考察

人工培地で培養した結核菌はトレハロースジミコール酸（trehalose dimycolate; TDM）を豊富に発現する。しかし、TDM は Mincle や TLR などの自然免疫受容体を介してマクロファージを活性化することから、宿主体内において結核菌は TDM の産生を抑制することにより自然免疫を回避する機構を有していることが想定された。TDM 合成の最終ステップにおいて、ミコール酸転移酵素の 2 つの基質結合部位にそれぞれトレハロースモノミコール酸（TMM）が結合し、一方の TMM（ドナー）から他方の TMM にミコール酸が転移されることにより TDM が産生される。一方、宿主体内にはグルコースが豊富に存在し、グルコースが TMM と拮抗的にミコール酸転移酵素のアクセプター基質となりうるため、GMM が産生されると同時に TDM の産生抑制が生じる。したがって、GMM は体内増殖菌のマーカー糖脂質となり、これに対する T 細胞応答の解明は、結核病

態や制御法の確立に重要であるとの考えから、本研究を展開した。

まず本研究により、GMM が遅延型アレルギー応答のアレルゲンとして機能することが初めて明らかになった。ツベルクリン反応に代表される古典的な遅延型アレルギー応答が MHC クラス 2 分子依存的であるのに対し、GMM に対する遅延型アレルギー応答は CD1 依存的であった。GMM はヒト CD1b 分子により T 細胞に提示されることが分かっているので、おそらくこの応答は CD1b 分子に拘束した反応であると推測できる。

本研究ではさらに、PPD に対する遅延型アレルギー応答と GMM に対する遅延型アレルギー応答は、拘束因子が異なるだけでなく、産生されるサイトカインプロファイルが異なることを実証した。PPD に対する応答は結核防御に重要な TH1 型サイトカイン応答だけでなくそれを抑制する TH2 型サイトカイン応答を伴っていたのに対し、GMM に対する応答は極度に TH1 サイトカイン応答にシフトしていた。このことは、GMM に対する遅延型アレルギー応答が結核防御に深く関与している可能性を示唆する。

ツベルクリン反応陽性が必ずしも結核防御免疫の成立を意味しないことは周知の事実である。GMM に対する皮内テストは TH1 型サイトカイン応答を忠実に反映することから、防御免疫のよりよい指標となりうることが考えられる。また GMM に対するメモリーT 細胞の存在が個体レベルで実証されたことから、GMM が脂質をベースとした新しいタイプの抗結核ワクチンとして有効である可能性も示唆される。したがって本研究により、結核の診断や予防の新しいパラダイムが確立されることが期待される。

E. 結論

体内増殖結核菌が特異的に産生する糖脂質に対して、TH1 型サイトカイン応答を主体とした遅延型アレルギー反応が惹起されることを示した。このことは、結核の診断やワクチン開発において、重要な示唆を与えるものである。

G. 研究発表

1. 論文発表

1) Komori, T., T. Nakamura, I. Matsunaga, D. Morita, Y. Hattori, H. Kuwata, N. Fujiwara, K. Hiromatsu, H. Harashima, and M. Sugita. 2011. A microbial glycolipid functions as a new class of target antigen for delayed-type hypersensitivity. J. Biol. Chem., 286: 16800-16806.

2. 学会発表

1) Sugita, M. Lipid-specific adaptive immunity in tuberculosis and AIDS. The 6th International Symposium of Institute Network, 9-10 June, 2011, Tokyo, Japan.

H. 知的財産権の出願・登録状況

1. 特許取得　　なし
2. 実用新案登録　なし
3. その他　　なし

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（国際医学協力研究事業）

抗酸菌における菌体構成成分の動態解析

分担研究報告書

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分担研究報告書

抗酸菌における菌体構成成分の動態解析

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研究要旨.

抗酸菌の病原性と深く関わる特徴の一つである細胞内寄生性は、そのメカニズムを含め未だ不明な点が多い。本研究では、抗酸菌の代謝機構がこの特徴に何らかの影響を与えているのではないかと想定し、抗酸菌の基礎代謝と関連する菌体内成分に注目した。結核菌、*M. bovis* BCG、*M. avium* complex 及び *M. smegmatis* から抽出した菌体内代謝成分を網羅的に解析し、そこに含まれる化合物の構成・量比を抗酸菌間で比較した。その結果、一部の代謝系関連物質の含有比が抗酸菌間で異なる傾向を示すことが判明した。さらに、細胞内寄生性との関連が指摘されているアミノ酸関連化合物が結核菌に偏在していることが明らかとなった。これらの結果は、抗酸菌の菌種間においても代謝系の仕組みに違いが存在することを示しており、病原性を含む抗酸菌の特徴に影響を及ぼしている可能性が示唆された。

A. 研究目的

抗酸菌は、結核やハンセン病に代表される人類にとって未だ大きな脅威となっている感染症を引き起こす原因細菌である。結核による死亡者は全世界で年間数百万人を数え、特にアジア等の発展途上国においても深刻な状況にある。一方、土壌や水中などの自然環境に広く存在し、結核様症状を誘導する非結核性抗酸菌症も近年増加傾向にあり同様に重要な慢性感染症である。結核菌（*Mycobacterium tuberculosis*）などは、宿主に感染後、病原性を発揮するために長期にわたって宿主細胞内で分裂増殖する。さらに、抗酸菌が細胞内での分裂増殖能を維持するためには、宿主内での一時的な生存だけでは不可能であり、細胞内の極めて偏った栄養環境を利用し得る特異な能力が不可欠であると考えられる。さらに、持続的な生育に関わる菌自身の代謝は、結核等の抗酸菌症の病態の推移・変化に影響を与えている可能性もある。しかしながら、これらのメカニズムについては未だ解明されていない。そこで、本研究では、抗酸菌自身の代謝に関連する成分に焦点を当て、それらの動態を解析することで細胞内寄生性との関連性を見出し、さらに、病態マーカーとなり得る成分の探索を行うことを目的とした。

B. 研究方法

実験には、結核菌は H37Rv 株、*M. bovis* BCG は Tokyo 株、*M. avium* complex は ATCC35767 株（4 型血清型）及び ATCC35771 株(8 型血清型)、さらに *M. smegmatis* mc^2155 株をそれぞれ使用した。培養は 7H9 液体培地（10% ADC 及び 0.05% Tween 80 含有）で行い、それぞれ 5.0×10^9 個の菌数を含む培養液を取得した。各抗酸菌菌体はフィルターを用いた吸引濾過により集菌し、Milli-Q 水による洗浄後、内部標準物質を含むメタノールによって菌体内部の成分を抽出した。クロロホルムによる脂質成分、さらに限外濾過フィルターによるタンパク質成分の除去をそれぞれ行い、乾固して解析用サンプルとした。解析には、CE-MS （capillary electrophoresis-mass spectrometry）法を採用し、サンプル中に含まれる候補化合物の種類等を検出ピークのスペクトルから予

測した。解析に先だって添加した内部標準物質及び各化合物の検出ピーク面積から、各化合物の含有比率を算出した。

倫理面への配慮　特になし

C. 研究結果

結核菌、*M. bovis* BCG、*M. avium* complex 4 型血清型、*M. avium* complex 8 型血清型及び *M. smegmatis* 由来サンプルより、合計 239 個の化合物が検出された。この内、90 個がカチオン（陽イオン性化合物）で、149 個がアニオン（陰イオン性化合物）であった。239 個の内、結核菌から 222 個、*M. bovis* BCG から 214 個、*M. avium* complex 4 型血清型から 213 個、*M. avium* complex 8 型血清型から 210 個、*M. smegmatis* から 224 個がそれぞれ検出された。さらに、193 個の化合物が 5 サンプル全てに認められた。内部標準物質に対する各化合物の含有比率を算出し抗酸菌間で比較した結果、化合物によっては数倍から数百倍の差が生じていた。これらの中から、解糖系、TCA サイクル、アミノ酸類及び核酸類について関連する化合物の含有比率を図 1 に示した。

D. 考察

本研究では、5 種の抗酸菌について、それぞれの菌体内成分の化合物構成・含有比を比較解析した。代謝系に当てはめた化合物群を比較した結果、解糖系、TCA サイクルにおいては著しい含有比の差は観察されなかった一方、dTDP や CDP といった多くの核酸系の成分において *M. smegmatis* での含有量が他の抗酸菌類に比較し亢進している傾向が認められた（図 1）。*M. smegmatis* は、本研究で解析した抗酸菌類の中で唯一の迅速生育タイプであり、核酸系代謝の亢進が迅速増殖・分裂という主要な形質と深く関わっている可能性がある。また、*M. avium* complex は二つの血清型株について解析を実施した。この二株は同じ菌種に属しながらも、細胞壁に含まれる一部の糖脂質の糖鎖構造が異なり、宿主に対する病原性も異なる性質を持つ。しかしながら、今回焦点を当てたほとんどの基礎代謝産物において、両株の間で含有量に大きな差が認められなかった（図 1）。つまり *M. avium* complex においては、病原性等に関わる機能の違いを生み出している要因は、菌体内成分よりむしろ細胞壁に存在する糖脂質分子などが多くを占めていることが推測される。一方、昨年度の研究において、*M. bovis* BCG とらい菌（*M. leprae*）との間にアミノ酸類の含有量に顕著な違いが見られたことを報告した。今回対象とした抗酸菌類は、らい菌以外の代表的なものであるが、アミノ酸類含有量において特徴的な差は観察されなかった（図 1）。このことは、らい菌が抗酸菌類の中でも特殊な代謝系を保持していることを間接的に示唆するものである。さらに、アミノ酸代謝の関連物質である betaine (trimethylglycine) が、結核菌に著しく偏在していており、その量比は他の抗酸菌に比べ百倍～数百倍であった（図 1）。Betaine については、結核菌においてその transporter が同定され、浸透圧調整や細胞内寄生に関与することが示されている。同様に *M. bovis* BCG も、非翻訳領域を含め塩基配列が結核菌 transporter 遺伝子と一致するゲノム領域を保持することから、本研究で判明した betaine の著しい差は未だ解明されていないその生合成に起因している可能性がある。また、結核菌における betaine の偏在は、病原性や診断法の観点からも興味深い点である。これまでは主に抗体産生を指標とした生化学的診断法が実施されてきたが、本研究で明らかになった菌体内成分の著しい差は、低分子化合物を標的とした新たな結核マーカー発掘の可能性を示唆するものである。このように、網羅的な菌体内成分の動態を解析することで、抗酸菌代謝系に関わる特徴の一端が明らかとなった。本研究では CE-MS 法により主に低分子イオン性化合物に焦点を絞ったが、今後さらに多くの成分を網羅し代謝系の全体像を掴むためには、化合物の化学的特性を考慮した複数のアプローチが必要となってくるであろう。

E. 結論

5種の抗酸菌類について、代謝に関連する菌体内低分子化合物を CE-MS 法により比較解析した結果、菌種間においても異なる特徴が存在し、さらに一部のアミノ酸関連成分が結核菌に偏在していることが判明した。

G. 研究発表

1. 論文発表

なし

2. 学会発表

1) Miyamoto, Y., M. Matsuoka, Y. Fukutomi, T. Mukai, M. Kai, Y. Maeda, and M. Makino. Characterization of intracellular metabolites from *Mycobacterium leprae*. International Union of Microbiological Societies 2011 Congress, 6-10 September, 2011, Sapporo, Japan.

2) Kai, M., H. Yamada, N. Fujiwara, S. Maeda, Y. Miyamoto, T. Mukai, N. Nakata, Y. Maeda, I. Yano, and M. Makino. Establishment and characterization of knockout mutants of *Mycobacterium bovis* BCG gene involved in mycolic acid. International Union of Microbiological Societies 2011 Congress, 6-10 September, 2011, Sapporo, Japan.

3) 向井 徹, 松岡正典, 前田百美, 宮本友司, 福富康夫, 牧野正彦. 蛍光蛋白発現らい菌構築のための検討. 第 84 回日本ハンセン病学会総会・学術大会 2011 年 5 月 岡山

4) 甲斐雅規, 松岡正典, 宮本友司, 中田登, 牧野正彦. 増殖能の異なるらい菌株間のゲノム比較解析. 第 84 回日本ハンセン病学会総会・学術大会 2011 年 5 月 岡山

H. 知的財産権の出願・登録状況

1. 特許取得 なし
2. 実用新案登録 なし
3. その他 なし

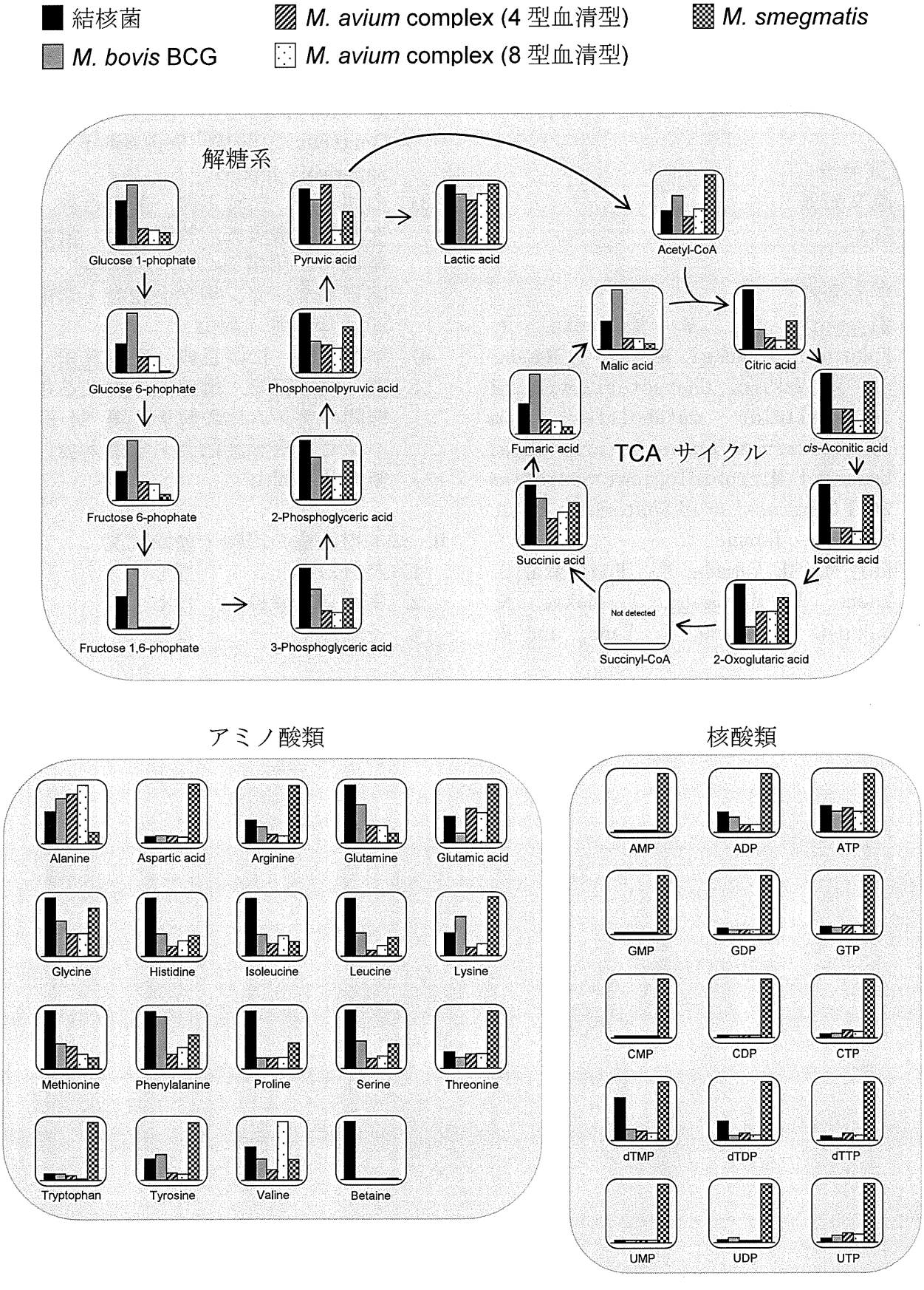

図 1. 解糖系-TCA サイクル、アミノ酸類及び核酸類の含有比率

研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Y. Tsukamoto, M. Endoh, T. Mukai, Y. Maeda, T. Tamura, M. Kai, M. Makino.	Immunostimulatory activity of major membrane protein II from *Mycobacterium tuberculosis*.	Clin. Vaccine Immunol.	18	235-242	2011
N. Nakata, M. Kai, M. Makino.	Mutation analysis of the *Mycobacterium leprae folP1* gene and Dapsone resistance.	Antimicrobial Agents and Chemotherapy	55	762-766	2011
S. Seto, K. Tsujimura, Y. Koide.	Rab GTPases regulating phagosome maturation are differentially recruited to mycobacterial phagosomes.	Traffic	12	407-420	2011
K. Sugaya, S. Seto, K. Tsujimura, Y. Koide.	Mobility of late endosomal and lysosomal markers on phagosomes analyzed by fluorescence recovery after photobleaching.	Biochem Biophys Res Commun.	410	371-375	2011
M. Okada, Y. Kita, T. Nakajima, N. Kanamaru, S. Hashimoto, T. Nagasawa, Y. Kaneda, S. Yoshida, Y. Nishida, H. Nakatani, K. Takao, C. Kishigami, S. Nishimatsu, Y. Sekine, Y. Inoue, D. N. McMurray, M. Sakatani.	Novel prophylactic vaccine using a prime-boost method and hemagglutinating virus of Japan-envelope against tuberculosis.	Clin. Dev. Immunol.		ID 549281 11 pages	2011
N. Pitabut. S. Mahasirimongkol, H. Yanai, C. Ridruechai, S. Sakurada, P. Dhepakson, P. Kantipong, S. Piyaworawong, S. Moolphate, C. Hansudewechakul, N. Yamada, N. Keicho, M. Okada, S. Khusmith.	Decreased plasma granulysin and increased interferon-gamma concentrations in patients with newly diagnosed and relapsed tuberculosis.	Microbiol. Immunol.	55	565-573	2011

H. Saiga, Y. Shimada, K. Takeda.	Innate immune effectors in mycobacterial infection.	Clin. Dev. Immunol.	2011	347594	2011
M. Takatsuka, M. Osada-Oka, E. F. Satoh, K. Kitadokoro, Y. Nishiuchi, M. Niki, M. Inoue, K. Iwai, T. Arakawa, Y. Shimoji, H. Ogura, K. Kobayashi, A. Rambukkana, S. Matsumoto.	A Histone-Like Protein of Mycobacteria Possesses Ferritin Superfamily Protein-Like Activity and Protects against DNA Damage by Fenton Reaction.	PLoS. One	6	e20985	2011
Y. Ozeki, Y. Hirayama, T. Takii, S. Yamamoto, K. Kobayashi, S. Matsumoto.	Loss of anti-mycobacterial efficacy in mice over time following vaccination with Mycobacterium bovis bacillus Calmette-Guerin.	Vaccine	29	6881-6887	2011
A. Takeuchi, T. Dejima, H. Yamada, K. Shibata, R. Nakamura, M. Eto, T. Nakatani, S. Naito S, Y. Yoshikai.	IL-17 production by γ δ T cells is important for the antitumor effect of Mycobacterium bovis bacillus Calmette-Guérin treatment against bladder cancer.	Eur. J. Immunol.	41	246-251	2011
K. Shibata, H. Yamada, T. Sato, S. Dejima, M. Nakamura, T. Ikawa, H. Hara, S. Yamasaki, R. Kageyama, Y. Iwakura, H. Kawamoto, H. Toh, Y. Yoshikai.	Notch-Hes1 pathway is required for the development of IL-17-producing γ δ T cells. Blood 118:586-93. 2011	Blood	118	586-593	2011
T. Komori, T. Nakamura, I. Matsunaga, D. Morita, Y. Hattori, H. Kuwata, N. Fujiwara, K. Hiromatsu, H. Harashima, M. Sugita.	A microbial glycolipid functions as a new class of target antigen for delayed-type hypersensitivity.	J. Biol. Chem.	286	16800-16806	2011

S. Daim, I. Kawamura, K. Tsuchiya, H. Hara, T. Kurenuma, Y. Shen, S. R. Dewamitta, S. Sakai, T. Nomura, H. Qu, M. Mitsuyama	Expression of the *Mycobacterium tuberculosis* PPE37 protein in *Mycobacterium smegmatis* induces low tumour necrosis factor alpha and interleukin 6 production in murine macrophages	J. Med. Microbiol.	60	582-591	2011
H. Kim, C. Nakajima, K. Yokoyama, Z. Rahim, Y. U. Kim, H. Oguri, Y. Suzuki.	Impact of the E540V amino acid substitution in GyrB of *Mycobacterium tuberculosis*on Quinolone Resistance.	Antimicrob. Agents Chemother.	55	3661-3667	2011
M. B. Hang' ombe, C. Nakajima, A. Ishii, Y. Fukushima, M. Munyeme, W. Matandiko, A. S. Mweene, Y. Suzuki.	Rapid detection of *Mycobacterium tuberculosis* complex in Cattle and Lechwe (*Kobus leche kafuensis*) at the slaughter house	Vet. Sci. Dev.	1	e5	2011
J. Wang, C. L. Zhang, L. Z. Zhang, B. Y. Ji, Y. Liu, Y. Z. Shao, S. L. Jiang, Y. Suzuki, C. Nakajima, C. L. Fan, Y. P. Ma, G. W. Tian, T. Hattori, H. Ling.	Genotypes and characteristics of clustering and drug-susceptibility of *Mycobacterium tuberculosis* isolates in Heilongjiang Province, China	J. Clin. Microbiol.	49	1354-1362	2011
Y. Fukutomi, Y. Maeda, M. Makino.	Apoptosis-inducing activity of clofazimine in macrophages.	Antimicrob Agents Chemother.	55	4000-4005	2011
Y. Horita, T. Takii, R. Kuroishi, T. Chiba, K. Ogawa, L. Kremer, Y. Sato, Y. Lee, T. Hasegawa, K. Onozaki	Synthesis and evaluation of anti-tubercular activity of new dithiocarbamate sugar derivatives.	Bioorg. Med. Chem. Lett.	21	899-903	2011

T. Tabuchi, T. Takatorige, Y. Hirayama, N. Nakata, S. Harihara, A. Shimouchi, K. Fujita, H. Yoshida, Y. Tamura, T. Nagai, T. Matsumoto, T. Takashima, H. Ito.	Tuberculosis infection among homeless persons and caregivers in a high-tuberculosis-prevalence area in Japan: a cross-sectional study.	BMC Infectious Diseases	11	22	2011
N. T. Hang, L. T. Lien, N. Kobayashi, T. Shimbo, S. Sakurada, P. H. Thuong, L. T. Hong, D. B. Tam, M. Hijikata, I. Matsushita, N. V. Hung, K. Higuchi, N. Harada, N. Keicho.	Analysis of factors lowering sensitivity of interferon-gamma release assay for tuberculosis.	PLoS One	6	e23806	2011
T. Tanaka, S. Sakurada, K. Kano, E. Takahashi, K. Yasuda, H. Hirano, Y. Kaburagi, N. Kobayashi, N. Hang, L. Lien, I. Matsushita, M. Hijikata, T. Uchida, N. Keicho.	Identification of tuberculosis-associated proteins in whole blood supernatant.	BMC Infect. Dis.	11	71	2011
Y. Maeda, T. Tamura, Y. Fukutomi, T. Mukai, M. Kai, M. Makino.	A lipopeptide facilitate induction of *Mycobacterium leprae* killing in host cells.	PLoS Neglected Tropical Diseases	in press		2011
T. Iwamoto, C. Nakajima, Y. Nishiuchi, T. Kato, S. Yoshida, N. Nakanishi, A. Tamaru, Y. Tamura, Y. Suzuki, M. Nasu.	Genetic diversity of *Mycobacterium avium* subsp. *hominissuis* strains isolated from human, pigs, and human living environment	Infect. Genet. Evol.	in press		2011

T. Naka, S. Maeda, M. Niki, N. Ohara, S. Yamamoto, I. Yano, J. I. Maeyama, H. Ogura, K. Kobayashi, N. Fujiwara.	The Lipid Phenotype of two Distinct Subpopulations of *Mycobacterium bovis* Bacillus Calmette-Guerin Tokyo 172 Substrain.	J. Biol. Chem.	in press		2011
N. Fujiwara, T. Naka, M. Ogawa, R. Yamamoto, H. Ogura H. Taniguchi.	Characteristics of *Mycobacterium smegmatis* J15cs strain lipids.	Tuberculosis	in press		2011

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1556-6811/11/$12.00 doi:10.1128/CVI.00459-10

Immunostimulatory Activity of Major Membrane Protein II from *Mycobacterium tuberculosis*▽‡

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Previously, we observed that both major membrane protein II of *Mycobacterium leprae* (MMP-ML) and its fusion with *M. bovis* BCG (BCG)-derived heat shock protein 70 (HSP70) (Fusion-ML) are immunogenic and that recombinant BCG secreting either of these proteins effectively inhibits the multiplication of *M. leprae* in mice. Here, we purified *M. tuberculosis*-derived major membrane protein II (MMP-MTB) and its fusion with HSP70 (Fusion-MTB) in a lipopolysaccharide-free condition and evaluated their immunostimulatory abilities. Both MMP-MTB and Fusion-MTB activated monocyte-derived dendritic cells (DC) in terms of phenotype and interleukin-12 (IL-12) production, but Fusion-MTB more efficiently activated them than MMP-MTB did. The IL-12 production was a consequence of the ligation of those recombinant proteins with Toll-like receptor 2. The *M. tuberculosis*-derived and *M. leprae*-derived recombinant proteins activated naïve T cells of both CD4 and CD8 subsets, but *M. tuberculosis*-derived proteins were superior to *M. leprae*-derived proteins and fusion proteins were superior to MMP, regardless of the origin of the protein. Memory-type CD4$^+$ T cells obtained from BCG-vaccinated healthy individuals seem to be primed with MMP-MTB by the vaccination, and both *M. tuberculosis*-derived recombinant proteins produced perforin-producing CD8$^+$ T cells from memory-type CD8$^+$ T cells. Further, infection of DC and macrophages with *M. tuberculosis* H37Ra and H37Rv induced the expression of MMP on their surface. These results indicate that *M. tuberculosis*-derived MMP, as a sole protein or as part of a fusion protein, may be useful for developing new vaccinating agents against tuberculosis.

Tuberculosis is a chronic infectious disease caused by intracellular infection with *Mycobacterium tuberculosis* (20). It is estimated that one-third of the global population is latently infected with this inhaled pathogen, which infects primarily macrophages and dendritic cells (DC), and tuberculosis is responsible for more than two million deaths yearly worldwide (11, 34, 36). The emergence of multidrug-resistant strains of *M. tuberculosis* mandates the development of more effective preventive and therapeutic strategies, including the development of improved vaccines (48). Protective immunity against *M. tuberculosis* is conducted chiefly by adaptive cellular immune responses, and gamma interferon (IFN-γ)-producing type 1 CD4$^+$ T cells and CD8$^+$ T cells are key components of this immunity (1, 12, 16). IFN-γ produced by activated T cells is believed to be an essential element of the host defense against *M. tuberculosis* (13). Further, the contribution of CD8$^+$ T cells to protection by lysing infected cells is also important for bacterial killing (7, 19). CD8$^+$ T cells can kill *M. tuberculosis*-infected host cells via a granule-dependent mechanism involving perforin and granulysin, which has a direct antimicrobial activity (42, 49).

The only approved vaccine currently available against tuberculosis is *M. bovis* bacillus Calmette-Guérin (BCG), an attenuated strain of *M. bovis*. More than four billion doses of BCG have been administered so far, and is established as a safe vaccine (29). BCG appears to be effective at preventing diseases such as tuberculous meningitis and miliary tuberculosis in newborns and toddlers; however, it has no apparent effect on pulmonary tuberculosis in adults (3, 9). The reason why BCG cannot prevent disease development is not fully known, but one of the reasons is based on the fact that BCG has a capacity to block phagosome maturation to inhibit antigen (Ag) processing and presentation to type 1 T cells (14, 32, 38). Indeed, although *M. tuberculosis* directly delivers Ag to the major histocompatibility complex (MHC) class I processing pathway, BCG was less able to activate CD8$^+$ T cells (35, 41). Further, BCG growing in human macrophages was not recognized by immune CD4$^+$ T cells, although BCG-infected macrophages continued to express MHC class II molecules (35). These observations indicate the need for the development of a new vaccine against tuberculosis.

Various new vaccine candidates which are based on Ags that are recognized in infected individuals are currently in clinical trials, including early secretory antigenic target 6 (ESAT-6), the Ag85 family, and a polyprotein Ag, designated Mtb72F, derived from *M. tuberculosis* proteins Mtb32 and Mtb59 (1, 2, 17, 18, 37, 39). However, a fully reliable new vaccine has not been established yet.

A situation similar to that of tuberculosis can be found in leprosy, which is caused by infection with *M. leprae*, and the development of a new vaccine capable of inhibiting the multi-

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plication of *M. leprae* is highly desirable. In both tuberculosis and leprosy, the activation of T cells is induced by DC loaded with bacilli or their components, which display various antigenic molecules on their surface, including the immunodominant Ags (15, 30), although there are conflicting results indicating that *M. leprae* inhibits the activation and maturation of DC (33). We are of the opinion that future vaccines, to be successful, must (i) be highly antigenic, (ii) have the capacity to activate both naïve $CD4^+$ T cells and $CD8^+$ T cells, and (iii) have the ability to be expressed on the surface of mycobacterium-infected Ag-presenting cells (APCs) such as macrophages and DC. Previously, we identified major membrane protein (MMP; gene name, *bfrA* or ML2038) as one of the immunodominant Ags of *M. leprae* (21). *M. leprae*-derived MMP (MMP-ML) ligates Toll-like receptor 2 (TLR2) and consequently activates the NF-κB pathway of host cells (21). DC pulsed with MMP-ML activate memory-type $CD4^+$ and $CD8^+$ T cells to produce IFN-γ in an Ag-specific fashion (21, 26). Further, MMP-ML is supposed to be recognized *in vivo* by T cells of *M. leprae*-infected individuals, including paucibacillary leprosy patients (26).

Further, when we introduced MMP-ML with the Ag85A secretion signal of *M. tuberculosis* into BCG, the modified BCG, termed BCG-SM, secreted MMP-ML, enhanced the ability of BCG to activate naïve $CD4^+$, and further, successfully activated naïve $CD8^+$ T cells (25). Furthermore, BCG-SM at least partially inhibited the growth of *M. leprae* in C57BL/6 mice subsequently challenged by injection in the footpads (22). These observations indicate that MMP-ML could be a target molecule to be further analyzed as a vaccine candidate, and the fact that BCG-SM can activate both subsets of naïve T cells to produce IFN-γ indicates that secretion of MMP-ML, presumably in the phagosome of APCs, is a useful strategy to activate T cells (25). We sought another strategy to further enhance the T cell-stimulating activity of BCG, especially of the ability to activate IFN-γ-producing $CD8^+$ T cells quickly and strongly. To this end, we used heat shock protein 70 (HSP70) as a fusion partner (6, 10, 44, 45). The gene encoding HSP70 of BCG was directly linked with that of MMP and extrachromosomally transformed into BCG (BCG-70 M) (31). BCG-70 M secreted the HSP70-MMP fusion protein (Fusion-ML) and activated not only Ag-specific naïve $CD8^+$ T cells polyclonally but also naïve $CD4^+$ T cells strongly (31). Further, the secreted Fusion-ML protein activated DC in terms of phenotype and the production of cytokines such as interleukin-12 (IL-12) (31). Thus, the production and secretion of HSP70 in phagosomes along with MMP-ML, using BCG as a vector, seem to be effective in activating human naïve $CD8^+$ T cells. These observations led us to speculate that the use of MMP, which is commonly present in pathogenic mycobacteria, or of the HSP70-MMP fusion protein may be useful in inhibiting the multiplication of *M. tuberculosis*. However, the MMP homology between *M. leprae* and *M. tuberculosis* (MMP-MTB; gene name, *bfrA* or Rv1876) is 90.6% at the amino acid level. Therefore, in this study, we purified *M. leprae*- or *M. tuberculosis*-derived MMP and a fusion protein composed of HSP70 and *M. leprae*- or *M. tuberculosis*-derived MMP by using *M. smegmatis* and evaluated their immunostimulatory activities.

MATERIALS AND METHODS

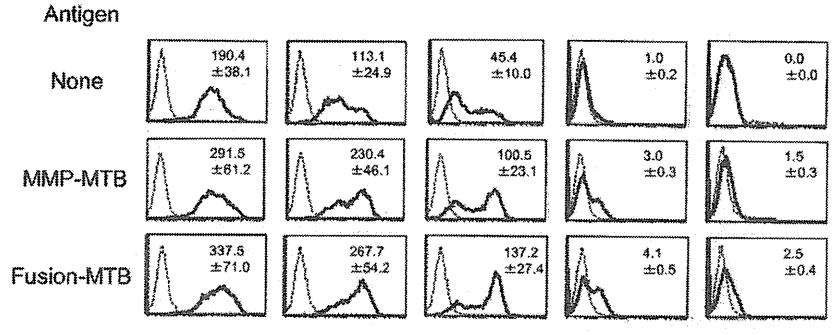
Preparation of cells and Ags. Peripheral blood was obtained from healthy, purified protein derivative-positive individuals after informed consent was obtained. In Japan, BCG vaccination is compulsory for children (0 to 4 years old). Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque Plus (Pharmacia, Uppsala, Sweden) and cryopreserved in liquid nitrogen until use, as previously described (23). The viability of T cells obtained from cryopreserved PBMCs was more than 90%, and no functional selection was induced in either monocytes or T cells by the cryopreservation of PBMCs. For the preparation of peripheral monocytes, $CD3^+$ T cells were removed from either freshly isolated heparinized blood or cryopreserved PBMCs using immunomagnetic beads coated with anti-CD3 monoclonal antibody (MAb; Dynabeads 450; Dynal Biotech, Oslo, Norway). The $CD3^-$ PBMC fraction was plated on collagen-coated plates, and the non-plastic-adherent cells were removed by extensive washing. The remaining adherent cells were used as monocytes (47). Monocyte-derived DC were differentiated as described previously (23, 28). Briefly, monocytes were cultured in the presence of 50 ng of recombinant granulocyte-macrophage colony-stimulating factor (rGM-CSF; PeproTech EC Ltd., London, England) and 10 ng of rIL-4 (PeproTech) per ml (28). On day 4 of culture, immature DC (purity, 70%) were pulsed with recombinant protein and on day 6 of culture, DC were used for further analyses of surface Ag and for mixed-lymphocyte assays. Macrophages were differentiated as described previously (24, 27). In brief, monocytes were cultured in the presence of 10 ng of rM-CSF (R&D Systems, Inc., Minneapolis, MN) per ml. On day 5 of culture, macrophages were pulsed with recombinant protein and on day 7 of culture, they were used for further analyses of surface Ag and for mixed-lymphocyte assays.

Preparation of *M. tuberculosis*. *M. tuberculosis* strains H37Ra and H37Rv, which were originally purchased from the American Tissue Culture Collection, were kindly donated by T. Yamazaki, National Institute of Infectious Diseases. Both H37Ra and H37Rv were cultured *in vitro* using Middlebrook 7H9 broth (BD Biosciences, San Jose, CA) supplemented with 0.05% Tween 80 and 10% albumin-dextrose-catalase (BD Biosciences). The bacilli were grown to log phase and stored at 10^8 CFU/ml at −80°C. Before the infection of DC and macrophages, H37Ra and H37Rv bacteria were counted by the colony assay method.

Construction of vectors for production of *M. leprae*- or *M. tuberculosis*-derived recombinant MMP and HSP70-MMP fusion protein (Fusion-ML) or Fusion-MTB. An *Escherichia coli-Mycobacterium* shuttle vector, pMV261 (43), was used as a parent vector. To replace the kanamycin resistance gene with a hygromycin resistance cassette, the XbaI-NheI fragment from pYUB854 (5) was cloned into SpeI-NheI-digested plasmids. The resultant vector was a hygromycin-resistant pMV261 vector, pMV261H. The acetamidase promoter was amplified from *M. smegmatis* mc^2155 genomic DNA. The primers used were F Pace Xba (5′-TTA ATC TAG AGA AGT GAC GCG GTC TCA AGC GTC-3′ [underlining indicates an XbaI site]) and R Pace Bam (5′-TTT AGG ATC CGT GGA CTC CCT TTC TCT TA-3′ [underlining indicates a BamHI site]). The *Hsp60* promoter region in pMV261H was replaced with the amplified PCR products, and the resulting vector was named p2H Pace.

We inserted an N-terminally His-tagged gene sequence encoding MMP-ML, MMP-MTB, HSP70-MMP-ML fusion (Fusion-ML), or HSP70-MMP-MTB (Fusion-MTB) into the p2H Pace vector using the In-Fusion Advantage PCR Cloning Kit (Clontech Laboratories, Inc.). Briefly, the linearized p2H Pace vector for the In-Fusion reaction was prepared by PCR with F ter (5′-TAG TTA ACT AGC GTA CGA T-3′) and R Pace H6 (5′-GTG ATG GTG GTG ATG GTG CAT GTG GAC TCC CTT TCT CTT AT-3′). PCR primers for inserts were designed that share 15 bases of homology with the sequences at the ends of linearized p2H Pace. These primers were used to amplify the insert DNAs for MMP-ML, MMP-MTB, Fusion-ML, and Fusion-MTB. The resulting PCR products were combined with the linearized vector in the In-Fusion cloning reaction and then transformed into *E. coli*. All clones was verified by sequencing.

Expression and purification of recombinant proteins in *M. smegmatis*. *M. smegmatis* mc^2155 was cultured *in vitro* using LB broth supplemented with 0.05% Tyloxapol (Sigma-Aldrich, St. Louis, MO). Expression vectors were introduced into *M. smegmatis* by electroporation (40). Transformants were selected on LB agar (BD Biosciences, San Jose, CA) plates containing 50 μg/ml hygromycin. The selected clone was grown in LB broth with 150 μg/ml hygromycin. During the logarithmic phase, acetamide was added to the culture medium at a final concentration of 0.2% (8). After an additional 16 h of culture, recombinant *M. smegmatis* was centrifuged and resuspended in lysis buffer (50 mM sodium phosphate, 300 mM NaCl, 3 M guanidine-HCl) containing proteinase inhibitor and disrupted by sonication. The suspension was centrifuged at 27,000 × *g* for 15 min. The supernatant was further filtered through a 0.45-μm filter and used as starting material. MMP-ML and MMP-MTB were purified by metal affinity



(p value)	HLA-ABC	HLA-DR	CD86	CD83	MMP
None vs MMP	p<0.05	p<0.05	p<0.05	p<0.001	p<0.05
None vs Fusion	p<0.05	p<0.05	p<0.05	p<0.005	p<0.01
MMP vs Fusion	p<0.05	p<0.05	p<0.005	p<0.05	p<0.005

FIG. 1. Expression of APC-associated molecules and MMP on DC by stimulation with recombinant proteins. Immature DC obtained from monocytes in the presence of rGM-CSF and rIL-4 were pulsed with either MMP-MTB or Fusion-MTB at 10 μg/ml on day 4 of culture. The DC were gated and analyzed on day 6 after the start of culture. Dotted lines, isotype-matched control IgG or IgM (for MMP); solid lines, the indicated test MAb. Representative results of three separate experiments are shown. The value in the top right corner of each graph is the mean fluorescence intensity of three independent experiments with a control Ig or the test MAb ± the standard deviation. Titers were statistically compared using Student's *t* test.

chromatography (TALON Metal Affinity Resins; Clontech Laboratories). Fusion-ML and Fusion-MTB were purified by two purification steps. Passage through a metal affinity column (TALON) was also used in the first step. The eluted crude proteins were applied to a HiLoad Superdex 200 pg column (GE Healthcare, Buckinghamshire, England) for further purification by gel filtration. Three major fractions were detected after the second step; one of them contained the target protein. The purified proteins (MMP-ML, MMP-MTB, Fusion-ML, and Fusion-MTB) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie brilliant blue staining (see Fig. S1 in the supplemental material).

Analysis of cell surface Ag. The expression of cell surface Ag on DC and lymphocytes was analyzed using a FACScalibur. Dead cells were eliminated from the analysis by staining with propidium iodide (Sigma-Aldrich), and 1×10^4 live cells were analyzed. For cell surface Ag analysis, fluorescein isothiocyanate (FITC)-conjugated MAbs against HLA-ABC (G46-2.6; BD Biosciences), HLA-DR (L243; BD Biosciences), CD86 (FUN-1; BD Biosciences), and CD83 (HB15a; Immunotech, Marseille, France) were used.

The expression of MMP on Ag-pulsed DC or DC infected with *M. tuberculosis* at an indicated multiplicity of infection (MOI) was determined using the MAb against MMP-ML (M270-13, IgM, kappa), which probably detects MMP complexed with MHC molecules on the surface of DC (26), followed by FITC-conjugated anti-mouse Ig Ab (Tago Immunologicals, Camarillo, CA). The intracellular production of perforin was assessed as follows. Memory-type $CD8^+$ T cells were stimulated with Ag-pulsed DC for 5 days in the presence of memory-type $CD4^+$ T cells, and $CD8^+$ T cells were surface stained with phycoerythrin-labeled MAb to CD8 and fixed in 2% formaldehyde. Subsequently, the cells were permeabilized using permeabilizing solution (BD Biosciences) and stained with an FITC-conjugated MAb to perforin (δG9; BD Biosciences) or an FITC-labeled isotype control.

APC functions of DC. The ability of Ag-pulsed DC and macrophages to stimulate T cells was assessed using an autologous APC-T cell coculture as previously described (15, 28). Purification of $CD4^+$ and $CD8^+$ T cells was conducted by using negative-isolation kits (Dynabeads 450; Dynal Biotech) (28). The purity of the $CD4^+$ and $CD8^+$ T cells was more than 95% as assessed by FACScalibur. Naïve $CD4^+$ and $CD8^+$ T cells were produced by further treatment of these T cells with MAb to CD45RO, which was followed by beads coated with MAb to goat anti-mouse IgG (Dynal Biotech). The purity of both subsets of naïve T cells was more than 97%. However, there was no contamination of memory-type T cells in the naïve T cell preparations. More than 98% of the $CD45RA^+$ T cells were positive for expression of the CCR7 molecule. Memory-type T cells were similarly produced by the treatment of cells with a MAb to CD45RA Ag. The purified responder cells (1×10^5 per well) were plated in 96-well round-bottom tissue culture plates, and DC or macrophages pulsed with Ag were added to give the indicated APC/T cell ratio. Supernatants of APC-T cell cocultures were collected on day 4, and the cytokine levels were determined. In some cases, Ag-pulsed DC were treated with MAbs to HLA-ABC (W6/32; mouse IgG2a, kappa), HLA-DR (L243; mouse IgG2a, kappa), CD86 (IT2.2; mouse IgG2b, kappa, BD Biosciences), or MMP (M270-13) or normal mouse IgG or IgM. The treatment of DC with these MAbs did not affect the viability of the DC (not shown). Also, in some cases, Ag-pulsed DC were costimulated with CD40 ligand (CD40L; 1 μg/ml; PeproTech). The optimal concentration was determined in advance.

Measurement of cytokine production. Levels of the following cytokines were measured: IFN-γ produced by $CD4^+$ and $CD8^+$ T cells and IL-12p40 produced by DC stimulated for 24 h with Ag. The concentrations of these cytokines were quantified with enzyme-linked immunosorbent assay (ELISA) kits (Opt EIA Human ELISA Set; BD Biosciences). The detection limit of the IFN-γ ELISA kit is 3.0 pg/ml.

Statistical analysis. Student's *t* test was used to determine statistically significant differences.

RESULTS

Activation of DC by *M. tuberculosis*-derived recombinant proteins. For a recombinant protein to activate T cells, it must have the ability to activate APCs. We assessed the phenotypic change induced in DC by stimulation with MMP-MTB (gene name, Rv1876 or *bfrA*) and a fusion protein composed of BCG-derived HSP70 and MMP-MTB (Fusion-MTB) (Fig. 1). Both recombinant proteins upregulated the surface expression of HLA-ABC, HLA-DR, CD86, and CD83. However, Fusion-MTB more efficiently enhanced the expression of all of these molecules. Further, MMP-MTB- or Fusion-MTB-pulsed DC expressed molecules which react with anti-MMP-ML MAbs. Again, Fusion-MTB was more efficient than MMP-MTB in the induction of expression of the molecules. These results indicated that both recombinant proteins may have the ability to activate DC. To confirm this point, we measured the IL-12p40 production of DC by stimulation with the recombinant proteins (Fig. 2). We comparatively analyzed MMP-ML, MMP-MTB, Fusion-ML, and Fusion-MTB. All of the recombinant proteins induced the production of IL-12p40, but the levels of IL-12p40 produced by stimulation were as follows: MMP-MTB > MMP-

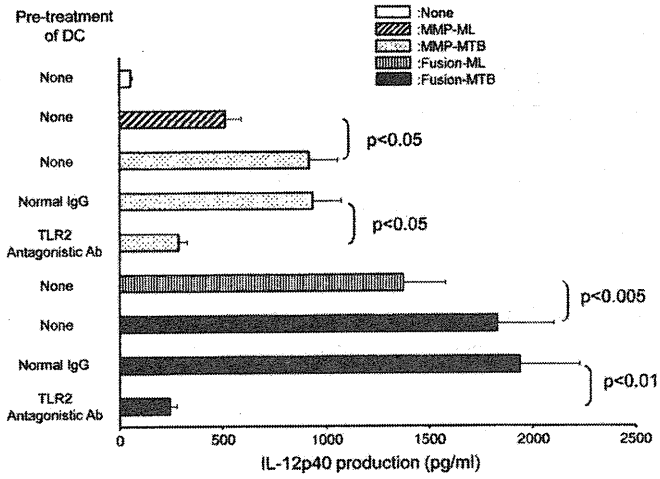


FIG. 2. IL-12p40 production by DC stimulated with recombinant proteins. Monocyte-derived DC from 5 days of culture in the presence of rGM-CSF and rIL-4 were stimulated with the indicated recombinant protein at 10 μg/ml for 24 h. In some cases, immature DC were pretreated with normal murine IgG or TLR2-antagonistic Ab (10 μg/ml) and subsequently stimulated with recombinant protein for 24 h. The concentration of IL-12p40 was determined by the ELISA method. A representative of three separate experiments is shown. Assays were performed in triplicate, and the results are expressed as the mean ± the standard deviation. Titers were statistically compared by Student's *t* test.

ML, Fusion-MTB > Fusion-ML, and Fusion-MTB > MMP-MTB. In order to reveal the mechanisms leading to the activation of DC by MMP-MTB or Fusion-MTB, we pretreated immature DC with TLR2-antagonistic Ab and subsequently stimulated the treated DC with recombinant proteins, since both MMP-ML and Fusion-ML are reported to activate the NF-κB pathway through ligation with TLR2 (21, 31). While pretreatment of DC with normal murine IgG did not affect the production of IL-12 by stimulation with recombinant proteins, the pretreatment with TLR2-antagonistic Ab significantly inhibited the cytokine production caused by MMP-MTB and Fusion-MTB (Fig. 2). Also, we tested the effect of TLR4-antagonistic Ab on IL-12 production; however, the pretreatment of DC with TLR4-antagonistic Ab did not inhibit cytokine production (not shown).

Activation of T cells by recombinant protein. The enhanced activation of DC by *M. tuberculosis*-derived MMP and fusion proteins may enable autologous T cell activation. The T cell-activating ability of the recombinant proteins was assessed chiefly by using DC as APCs. Memory-type $CD4^+$ T cells were purified from healthy, BCG-vaccinated individuals. All of the recombinant proteins activated the $CD4^+$ T cells with a small dose (~2.0 μg/ml) of Ags and a small dose of recombinant protein-pulsed DC (T cell/DC ratio, 80:1) (Fig. 3A). However, MMP-MTB and Fusion-MTB more efficiently activated the T cells than *M. leprae*-derived proteins did, and Fusion-MTB induced a significantly larger amount of IFN-γ than MMP-MTB did. When we used a higher concentration of recombinant proteins, such as 5 or 10 μg/ml, and used a higher number of DC, such as a T cell/DC ratio of 10:1, as a stimulator, similar statistically significant differences were observed (not shown). Further, only Fusion-MTB successfully activated memory-type $CD4^+$ T cells through macrophages, although a large dose of Ag and a high number of Ag-pulsed macrophages were required (T cell/macrophage ratio, 5:1) (Fig. 3A). The $CD4^+$ T cell-stimulating activity of MMP-MTB and Fusion-MTB was confirmed by using CD45RO-negative naïve $CD4^+$ T cells. All of the recombinant proteins, including MMP-ML, MMP-MTB, Fusion-ML, and Fusion-MTB, activated naïve $CD4^+$ T cells, and Fusion-MTB was the most effective (Fig. 3B). Compared to memory $CD4^+$ T cells, naïve $CD4^+$ T cells required a larger dose of Ag (~10 μg/ml) and a higher number of Ag-pulsed DC (T cell/DC ratio, 10:1) to be activated. To address the mechanisms leading to the activation of naïve $CD4^+$ T cells by Fusion-MTB, Fusion-MTB-pulsed DC were treated with MAbs against HLA-DR, CD86, and MMP-ML molecules and subsequently used to stimulate naïve $CD4^+$ T cells (Fig. 3C). IFN-γ production by these naïve $CD4^+$ T cells was significantly inhibited by the surface treatment of the DC with the MAbs, and similarly, IL-2 production by naïve $CD4^+$ T cells was inhibited (not shown). The ability of MMP-MTB and Fusion-MTB to activate memory-type $CD8^+$ T cells was then assessed (Fig. 4A). Although, in contrast to memory-type $CD4^+$ T cells, a large dose of recombinant proteins was required, both *M. tuberculosis*-derived recombinant proteins induced significant production of IFN-γ from memory-type $CD8^+$ T cells. Further, the additional treatment of Ag-pulsed DC with CD40L upregulated the production of IFN-γ by $CD8^+$ T cells. In both cases, i.e., without and with CD40L treatment, Fusion-MTB induced significantly greater IFN-γ production than MMP-MTB did. In order to confirm the $CD8^+$ T cell-stimulating abilities of both MMP-MTB and Fusion-MTB, naïve $CD8^+$ T cells were also examined as responders. In this case, purified proteins from *M. leprae* were used as a control (Fig. 4B). Both MMP-MTB and Fusion-MTB activated naïve $CD8^+$ T cells to produce IFN-γ; however, the concentration of IFN-γ released from naïve $CD8^+$ T cells was low and a cytokine concentration of less than 35 pg/ml was produced, and the concentration of IFN-γ produced from naïve $CD8^+$ T cells by stimulation with Fusion-MTB was significantly lower than that from memory $CD8^+$ T cells ($P < 0.005$). The naïve $CD8^+$ T cell-stimulating activities of the recombinant proteins were as follows: MMP-MTB > MMP-ML, Fusion-MTB > Fusion-ML, and Fusion-MTB > MMP-MTB. The IFN-γ production by naïve $CD8^+$ T cells was enhanced by the additional treatment of Ag-pulsed DC with CD40L, and the highest production of IFN-γ was achieved by Fusion-MTB; in this case, Fusion-MTB could induce an IFN-γ concentration of more than 100 pg/ml. To elucidate the mechanisms of the activation of naïve $CD8^+$ T cells by Fusion-MTB, Fusion-MTB pulsed DC were treated with MAbs to HLA-ABC and CD86 and subsequently used as a stimulator (Fig. 4C). IFN-γ production by naïve $CD8^+$ T cells was significantly inhibited by the treatment of the DC. One of the aims of $CD8^+$ T cell activation in terms of the host defense against *M. tuberculosis* is to produce cytotoxic $CD8^+$ T cells. To measure the production of cytotoxic $CD8^+$ T cells, we assessed the intracellular production of perforin in $CD8^+$ T cells which were stimulated with MMP-MTB or Fusion-MTB in the presence of $CD4^+$ T cells (Fig. 4D). Both recombinant proteins produced perforin-producing $CD8^+$ T cells, and Fusion-MTB seemed to produce them more efficiently.

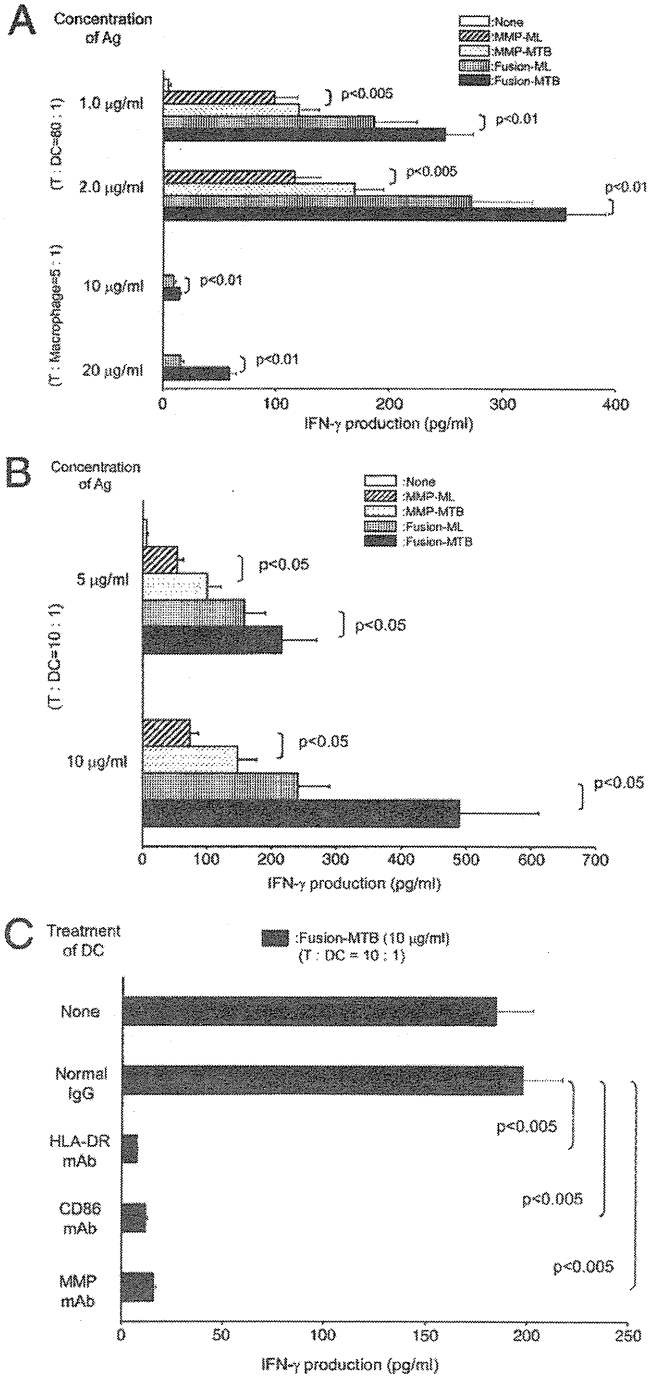


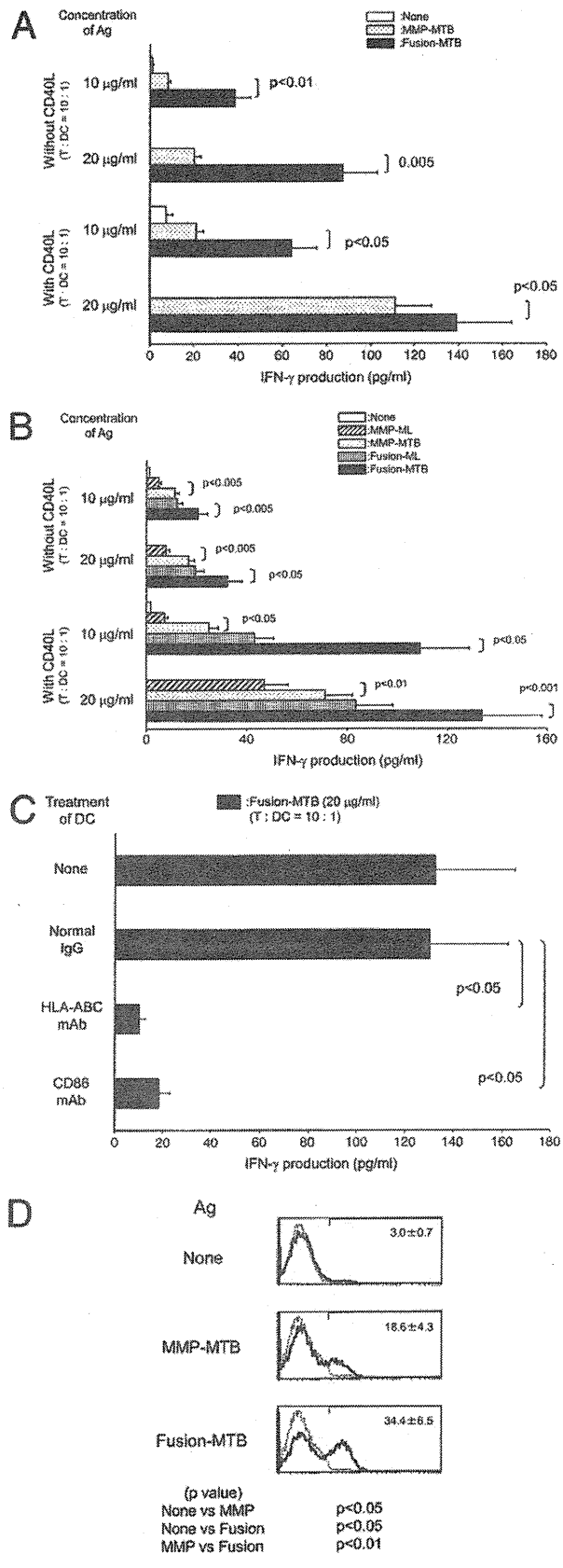
FIG. 3. (A) IFN-γ production by memory-type $CD4^+$ T cells stimulated with recombinant proteins. Monocyte-derived DC or macrophages were pulsed with the indicated recombinant protein at the indicated concentration and used to stimulate memory-type $CD4^+$ T cells in a 4-day culture. Responder $CD4^+$ T cells (1×10^5) were stimulated with the indicated dose of Ag-pulsed DC or macrophages. (B) IFN-γ production by naïve $CD4^+$ T cells by stimulation with recombinant protein. Monocyte-derived DC were pulsed with the indicated recombinant protein at 5 or 10 μg/ml and used to stimulate naïve $CD4^+$ T cells in a 4-day culture. Responder $CD4^+$ T cells (1×10^5) were stimulated with the Ag-pulsed DC at a T cell/DC ratio of 10:1. (C) Inhibition of naïve $CD4^+$ T cell activation by treatment of Ag-pulsed DC with MAb. Monocyte-derived DC were pulsed with Fusion-MTB at 10 μg/ml and subsequently treated at 10 μg/ml with MAb to HLA-DR, CD86, MMP, or normal murine IgG or IgM. These DC were used to stimulate naïve $CD4^+$ T cells (1×10^5) at a T cell/DC ratio of 10:1. IFN-γ produced from T cells was measured by the ELISA method. A representative of three separate experiments is shown. Assays were performed in triplicate, and the results are expressed as means ± standard deviations. Titers were statistically compared by Student's *t* test.

Expression of MMP on APCs infected with *M. tuberculosis*. The molecule used as a vaccinating agent should be expressed on APCs infected with the pathogen. To reveal the expression of MMP on the surface of APCs, DC and macrophages were infected with *M. tuberculosis* H37Ra and H37Rv and analyzed by flow cytometry (Fig. 5). Both DC and macrophages expressed MMP molecules on their surface after infection with H37Ra and H37Rv. Expression levels seemed to be dependent on the dose of *M. tuberculosis* used for infection (not shown).

DISCUSSION

In vivo studies using various knockout mice indicate that adaptive immunities play an important role in inhibiting the multiplication of *M. tuberculosis* and that the activation of both $CD4^+$ T cells and $CD8^+$ T cells is an essential element of the control of *M. tuberculosis* infection (1, 12, 16). While $CD4^+$ T cells chiefly act in the initial phase of infection, $CD8^+$ T cells either producing IFN-γ or having cytotoxic killing activity contribute to the chronic or stationary phase of infection (7, 19, 46). Thus, the antigenic molecules which are used as an essential component of a vaccine should have the ability to activate not only naïve $CD4^+$ T cells and $CD8^+$ T cells but also APCs, including DC. So far, we have found MMP to be one of the immunodominant Ags of *M. leprae* (21) and found evidence that MMP-ML activated DC through ligation with TLR2, which resulted in the activation of the NF-κB pathway of host cells, and that DC pulsed with MMP-ML stimulated both $CD4^+$ and $CD8^+$ T cells to produce IFN-γ in an Ag-specific manner (21, 26). Further, MMP-ML is supposed to be recognized *in vivo* by both T cell subsets of *M. leprae*-infected individuals, including paucibacillary leprosy patients (26).

In addition, HSP70, one of the heat shock proteins, plays various roles in the upregulation of the ability of APCs to stimulate T cells (6, 10, 44, 45). Further, HSPs of both mammalian host cell and bacterial origins are reported to have chaperon activity (6, 44) and can effectively prime a cytolytic response (10, 45). In fact, we previously reported that HSP70 effectively induced the cross-priming of $CD8^+$ T cells through the cytosolic pathway when secreted from recombinant BCG in the phagosome of DC as part of a fusion protein (31). Also, others have reported that HSP65 activated naïve $CD8^+$ T cells and a DNA vaccine containing the *hsp65* gene inhibited the development of tuberculosis that is induced by the multiplication of subsequently challenged *M. tuberculosis* (50). Furthermore, vaccination of mice with recombinant BCG that secreted either MMP-ML or Fusion-ML, in which BCG was used as a vehicle, efficiently inhibited the multiplication of subsequently challenged *M. leprae*, although the fusion protein was more efficient in both activating naïve T cells and inhibiting *M. leprae* multiplication (22, 25, 31).



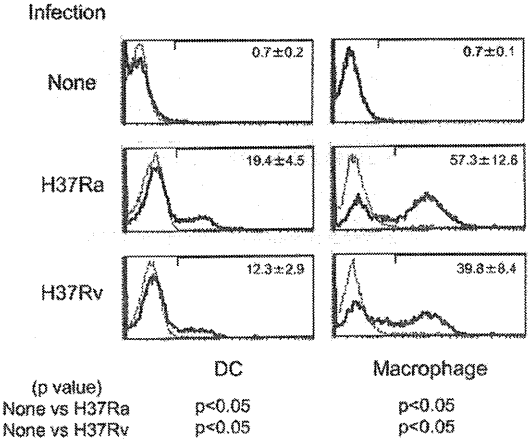


FIG. 5. Expression of MMP on DC and macrophage infected with *M. tuberculosis*. Monocyte-derived DC or macrophages were infected with either H37Ra or H37Rv at an MOI of 1.0 and cultured for another 2 days in the presence of rGM-CSF plus rIL-4 or rM-CSF, respectively. The DC and macrophages were gated and analyzed on day 5 after the start of culture. Dotted lines, control IgM; solid lines, MMP MAb. Results representative of three separate experiments are shown. The values are the mean percentages of major membrane protein II-positive DC or macrophages in three independent experiments and the standard deviations. Titers were statistically compared using Student's *t* test.

MMP is commonly expressed in both pathological mycobacteria and BCG, so that it may be that MMP-MTB plays a substantial role in inhibiting the replication of *M. tuberculosis*; however, the homology of MMP between *M. leprae* (ML2038)

FIG. 4. (A) IFN-γ production by memory-type $CD8^+$ T cells by stimulation with recombinant protein. Monocyte-derived DC were pulsed with MMP-MTB or Fusion-MTB at 10 or 20 μg/ml, costimulated with or without CD40L (1.0 μg/ml), and used to stimulate memory-type $CD8^+$ T cells in a 4-day culture. Responder $CD8^+$ T cells (1×10^5) were stimulated with the Ag-pulsed DC at a T cell/DC ratio of 10:1. (B) IFN-γ production by naïve $CD8^+$ T cells stimulated with recombinant proteins. Monocyte-derived DC were pulsed with the indicated recombinant protein at 10 or 20 μg/ml, further costimulated with or without CD40L (1.0 μg/ml), and used to stimulate naïve $CD8^+$ T cells in a 4-day culture. Responder $CD8^+$ T cells (1×10^5) were stimulated with the Ag-pulsed DC at a T cell/DC ratio of 10:1. (C) Inhibition of naïve $CD8^+$ T cell activation by treatment of Fusion-MTB-pulsed DC with MAb. Monocyte-derived DC were pulsed with MMP-MTB at 20 μg/ml, costimulated with CD40L (1.0 μg/ml), and subsequently treated at 10 μg/ml with MAb to HLA-ABC, CD86, or normal murine IgG. These DC were used to stimulate naïve $CD8^+$ T cells (1×10^5) at a T cell/DC ratio of 10:1. IFN-γ produced by T cells was measured by the ELISA method. A representative of three separate experiments is shown. Assays were performed in triplicate, and the results are expressed as means ± standard deviations. Titers were statistically compared by Student's *t* test. (D) Intracellular production of perforin by $CD8^+$ T cells. Monocyte-derived DC were pulsed at 10 μg/ml with either MMP-MTB or Fusion-MTB and cultured with unseparated memory-type T cells (T cell/DC ratio, 40:1) for 5 days. The stimulated $CD8^+$ T cells were gated and analyzed for perforin production. Values are the mean percentages of the $CD8^+$ T cell population that were perforin positive in three independent experiments and the standard deviations. Titers were statistically compared using Student's *t* test. A representative of three separate experiments is shown.