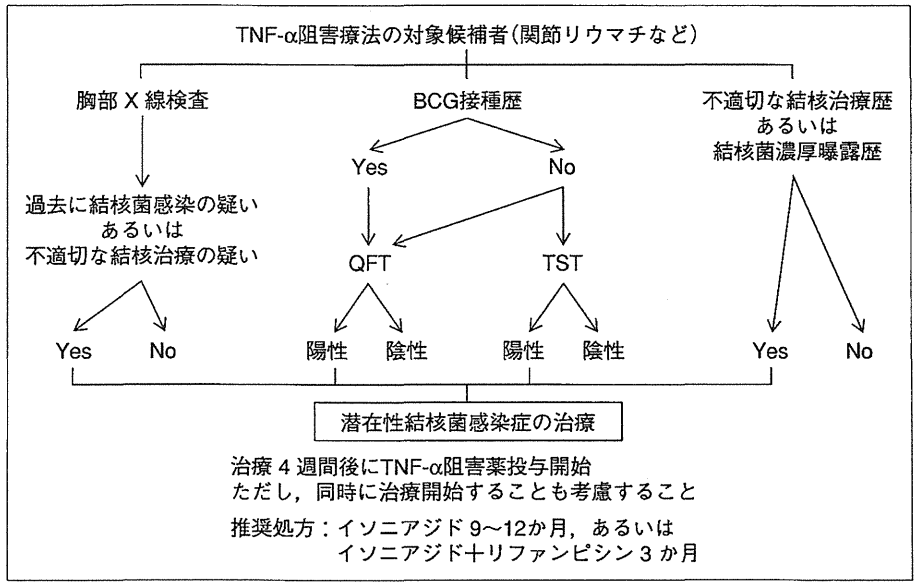

図2　潜在性結核菌感染の検査および治療法(文献[16]より引用改変)

の報告がある．TST陽性者，特に反応の強い者は活動性結核への進行リスクが高いこと，TST陽性者の治療は活動性結核の発症リスクを減少させることなどである[10)~13)]．これに対して，QFTではこれに匹敵するデータがない．

近年，関節リウマチやクローン病など免疫疾患の治療に使用されるTNF-α阻害薬は潜在性結核菌感染から内因性再燃機序により活動性結核の発症リスクを増強することが報告されている[14)~16)]．CDCは，TNF-α阻害薬使用前に潜在性結核菌感染の有無を確認し，感染があれば積極的に抗結核化学療法(イソニアジド，リファンピシン)の施行を推奨している[17)]．検査は胸部X線検査，BCG接種歴があればQFT，なければQFTとTST，および結核菌感染歴・曝露歴の確認が必要であり，検査陽性の場合，結核治療開始4週間後からTNF-α阻害薬を使用することが推奨されている(図2)．TNF-α阻害薬使用者だけでなく，HIV感染者，透析患者，糖尿病患者，副腎皮質ステロイド薬使用者も免疫抑制状態にあり，活動性結核の発症リスクが高く，発症の多くは潜在性結核菌感染からの内因性再燃による．一方，免疫抑制状態にある患者では，QFT応答が抑制され，結核菌感染していても陰性または判定不可となる可能性がある．しかし，TSTよりもQFTの方が応答の抑制程度が軽く，高い感度が維持されているため，ある程度感度が低下している可能性を念頭におきながら，QFTを実施すべきである．

非結核性抗酸菌感染症の診断

1．非結核性抗酸菌の特徴

非結核性抗酸菌(nontuberculous mycobacteria；NTM，非定型抗酸菌：atypical mycobacteria, mycobacteria other than tuberculosis；MOTT, potentially pathogenic environmental mycobacteria；PPEM)は結核菌群とらい菌を除いた抗酸菌である．非結核性抗酸菌は環境菌であり，水，土壌や動物に普遍的に存在する．多くの場合，健常者に対して病原性を示すことは少ない．また，ヒト—ヒト感染はなく，感染症法の対象外であり隔離は不要である．しかしながら，結核を含む抗酸菌感染症の約10～20％を非結核性抗酸菌感染症が占め(世界：100～200万人，日本：2,500～5,000人/年)，非結核性抗酸菌感染症は日本を含め世界的に増加している．実際，米国では非結核性抗酸菌感染症患者数は結核患者数を凌駕している．

非結核性抗酸菌による肺感染症の原因菌として，*Mycobacterium avium* complex(*M. avium*と*M. intracellulare*は細菌学的にきわめて類似している

表5 主要な非結核性抗酸菌感染症の特徴

菌種	頻度	至適温度	感染源，経路	抗菌薬感受性
M. avium complex	最頻(70%)	37℃	水，土壌，鳥類	耐性
M. kansasii	頻(20%)	37℃	水，土壌	感受性
M. marinum	少ない	30℃	水，土壌	感受性
M. ulcerans(Buruli潰瘍)	頻(アフリカ，オセアニア)	30℃	経皮感染，動物由来？	耐性

表6 非結核性抗酸菌感染症と原因菌

疾患	主要な病原体
肺感染症	*M. avium, M. intracellulare, M. kansasii, M. abscessus, M. xenopi*
リンパ節炎	*M. avium, M. intracellulare, M. scrofulaceum*
皮膚感染症	*M. marinum, M. fortuitum, M. chelonae, M. abscessus, M. ulcerans*
播種性感染症	*M. avium, M. intracellulare, M. kansasii, M. chelonae, M. abscessus, M. haemophilum*

表7 肺MAC感染症の診断基準(米国胸部疾患学会・感染症学会，2007)

1)臨床所見(①+②)
　①呼吸器症状(咳嗽や喀痰)，単純胸部X線で結節あるいは空洞病変，あるいは高解像度CTによる多発性小結節を伴う多巣性気管支拡張
　②他疾患の除外
2)微生物学的所見(①～③のいずれか)
　①複数の喀痰検体で2回以上の培養陽性
　②気管支洗浄液で1回以上の培養陽性
　③経気管支内視鏡下あるいは開胸肺生検で肉芽腫炎症，あるいは抗酸菌陽性，あるいは1回以上の培養陽性

(文献[18]より引用改変)

ため，一括して*Mycobacterium avium* complex；MACと表す)が70～80%，*M. kansasii*が20%を占める(表5)．リンパ節炎はMACや*M. scrofulaceum*，皮膚感染症は*M. marinum*(魚槽肉芽腫fish tank granulomas)，*M. fortuitum*，*M. chelonae*，*M. abscessus*や*M. ulcerans*(Buruli潰瘍)，重度の免疫不全の場合，播種性感染症はMAC，*M. kansasii*，*M. chelonae*，*M. abscessus*や*M. haemophilum*などが多い(表6)．

非結核性抗酸菌感染症の好発要因として，進行したAIDS(末梢血CD4陽性T細胞数≦100/μL)など免疫不全，肺基礎疾患(気管支拡張症，肺嚢胞，塵肺や陳旧性結核など)やTNF-α阻害療法が知られているが，これらの状況を欠如した症例もしばしば認められる．MACは多くの抗結核薬に耐性を示すことが多く，比較的有効な新規マクロライド系抗菌薬(クラリスロマイシンやアジスロマイシン)が用いられているが，根治は困難である．なお，*M. kansasii*は通常の抗結核薬に感受性を示すことが多い．

2．非結核性抗酸菌感染症の診断

非結核性抗酸菌感染症の診断は米国胸部疾患学会・感染症学会の診断基準(2007)による[18]．その骨子として，1)臨床所見として①呼吸器症状(咳嗽や喀痰)があり，単純胸部X線で結節あるいは空洞病変が認められる，あるいは高解像度CTによる多発性小結節を伴う多巣性気管支拡張が認められる，および②他疾患が除外される条件を満たし，2)微生物学的所見として①複数の喀痰検体で2回以上の培養陽性，②気管支洗浄液で1回以上の培養陽性，③経気管支内視鏡下あるいは開胸肺生検で肉芽腫炎症，あるいは抗酸菌陽性，あるいは1回以上の培養陽性のいずれかの条件を満たすことが基準となる(表7)．しかし，臨床と微生物所見を加味するため，確定診断に少なくとも1か月を要することから，迅速かつ簡便な診断方法が望まれている．

MAC特異的細胞壁糖ペプチド脂質(GPL)は分子量約1.2kDaの31血清型MACに存在し，かつ，主要抗原である．遅発育菌であるMACおよび*M.*

*scrofulaceum*や迅速発育菌である*M. chelonae*および*M. fortuitum*はGPLを保有するが，結核菌群(BCGを含む)は保有しない．そこで抗GPL-IgA抗体検出によるMAC感染症の迅速血清診断法(所要時間3時間)が開発された[19][20]．活動性MAC感染症の血清診断に関し，多施設共同研究の結果として，喀痰培養陽性MAC患者の血清診断感度は米国胸部疾患学会・感染症学会の診断基準(2007)に比較し，84.3%，特異度は100%であり，喀痰培養陰性(気管支肺胞洗浄液培養陽性)MAC患者の感度は73.6%，特異度は96.5%であった．MAC-GPL抗原を用いた血清抗体検出は活動性MAC感染症の非侵襲性で安全な迅速診断法として有用であるため，厚生労働省からMAC-GPL診断キットの体外診断用医薬品製造販売が承認され，2011年8月22日に保険収載，臨床応用が開始されている(キャピリア®MAC抗体ELISA，タウンズ)．今後，MAC-GPL診断キットの市販後性能評価が進み，診断基準に付加されることが望まれる．

結　語

感染症診療や対策において，迅速，簡便かつ正確な診断は重要であり，感染の蔓延防止，治療や予防に寄与する．既存の診断法に加え，今後，Xpert® MTB/RIFやMAC-GPL診断キットなど，革新的な迅速診断が活動性結核や非結核性抗酸菌感染症対策に貢献することが期待される．多くの抗酸菌感染症は潜在性抗酸菌感染からの内因性再燃に起因しているが，潜在性抗酸菌感染症(潜在性結核菌感染および潜在性MAC感染)の診断に関する“gold standard”はなく，その研究開発は急務の課題である．

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解説
結核や非結核性抗酸菌感染症の動向と最近の話題

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要 旨

結核はヒト免疫不全ウイルス（HIV）感染症／後天性免疫不全症候群（AIDS）、マラリアと共に世界3大感染症である。世界で約20億人（総人口の約1/3、日本：0.25億人）が結核菌（*Mycobacterium tuberculosis*）に無症候・潜在性既感染、年間880万人（日本：2.3万人）が結核を発病、140万人（日本：0.22万人）が死亡し、結核は甚大な健康被害を与え続けている（2011年）。結核菌類縁の非結核性抗酸菌（nontuberculous mycobacteria：NTM）は結核菌群とらい菌を除いた抗酸菌群である。結核を含む抗酸菌感染症の約 10-20%を NTM 感染症（世界：100-200万人、日本：2,500-5,000人/年）が占め、NTMの内訳として、最頻は*Mycobacterium avium* complex（MAC）である。NTM感染症は日本を含め世界的に増加している。実際、アメリカ合衆国ではNTM感染症患者数は結核患者数を凌駕している。本稿では、結核やNTM感染症の動向や最近の革新的な話題（結核菌遺伝子全自動検出系、日本発新規抗結核薬やMAC感染症の血清診断）について概説する。

結 核

（biosafety level：BSL-3病原体、四種病原体、多剤耐性結核菌は三種病原体）

結核（感染症法二類感染症）は結核菌（biosafety level：BSL-3病原体、四種病原体、多剤耐性結核菌は三種病原体）を含む飛沫核（空気感染）により伝播する感染症である。結核の集団感染や施設内感染はしばしばであり、感染管理として空気感染予防策が重要である（表1）。また、実験室感染の原因病原体として、結核菌は最頻であり、BSL-3における取扱いが必須である。

結核菌暴露者が、飛沫核を吸入し、結核菌が肺胞内に定着し、全身に広がることで感染が成立する（暴露者の約30%）。また、感染者の約10%が活動性結核を発症するが、残りの約90%では感染した結核菌は休眠状態（dormancy）、すなわち、無症候潜在感染状態で宿主の体内にとどまる（潜在性結核菌感染）（図1）。潜在感染した結核菌は、老化、免疫抑制療法（免疫抑制薬、ステロイド薬、抗サイトカイン療法：腫瘍壊死因子-α阻害薬など）、栄養障害やHIV感染/AIDSなどの宿主免疫機能の低下により、発育、増殖を再開し、活動性結

表1. 空気感染と予防策（Airborne precautions）

空気（飛沫核）感染：	微粒子（≤5 μm）により伝播する感染症、患者と空気を共有することにより感染する	
空気（飛沫核）感染症：	麻疹、水痘、結核	
結核の感染管理：	感染源対策	早期発見、隔離、治療
	感染経路対策	菌密度の減少（換気、紫外線照射、N-95マスク）
	感受性宿主対策	発病の予防（BCG接種：生後6カ月以内、化学予防：成人）
	個室収容	陰圧、換気（7-8回／毎時）、独立空調、HEPAフィルター
	実験室	BSL-3
	空気感染予防策	標準的予防策＋高性能（N-95）マスク着用

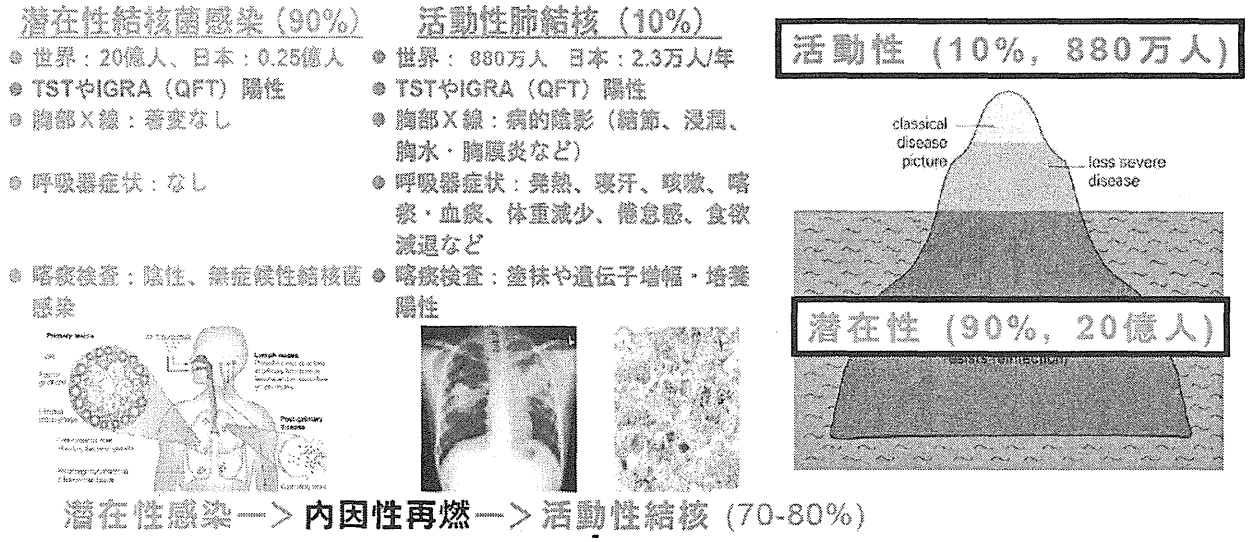


図１．潜在性結核と活動性結核
（引用：Nat. Rev. Microbiol. 1: 97-105, 2003）

核を発症する（内因性再燃）。成人の場合、潜伏感染の内在性再燃により活動性結核を発症することが多い（約70%）。他方、小児や日和見宿主（HIV感染症/AIDSや免疫抑制療法など）では、初感染によって活動性結核を発症しうる。

発生動向とし、世界で約20億人（総人口の約1/3、日本：0.25億人）が結核菌（*Mycobacterium tuberculosis*）に無症候・潜在性既感染、年間880万人（日本：2.3万人）が結核を発病、140万人（日本：0.22万人）が死亡し、結核は甚大な健康被害を与え続けている（2011年）（1, 2）。都市化による過密、人口の集中、貧困（disease of poverty）、交通機関の発達による人民の高速移動、国際化、環境破壊や温暖化など、現代社会の直面している状況が感染症の増加に関与している。

世界に共通した対策の課題として、１）薬剤耐性結核（特に、isoniazidとrifampicinに同時耐性を示す多剤耐性結核、約65万人、日本：300人）や２）HIV-結核菌の重複感染（新規発生患者：13%、日本：0.3%）が重要である（3）。

非結核性抗酸菌感染症
（ほとんどの非結核性抗酸菌はBSL-2、一部：BSL-1）

非結核性抗酸菌（nontuberculous mycobacteria：NTM、非定型抗酸菌：atypical mycobacteria、mycobacteria other than tuberculosis：MOTT、potentially pathogenic environmental mycobacteria：PPEM）は結核菌群とらい菌を除いた抗酸菌である。NTMは環境菌であり、水、土壌や動物に普遍的に存在する。多くの場合、健常者に対して病原性を示すことは少ない。また、感染症法の対象外であり、ヒト—ヒト感染はなく、届出や隔離は不要である。結核を含む抗酸菌感染症の約10-20%をNTM感染症が占め（世界：100-200万人、日本：2,500-5,000人/年）、加えて、NTM感染症は日本を含め世界的に増加している。実際、アメリカ合衆国ではNTM感染症患者数は結核患者数を凌駕している（4）（表2）。

非結核性抗酸菌による肺感染症の原因菌として、*Mycobacterium avium* complex（*M. avium* と *M. intracellulare* は細菌学的に極めて類似しているため、MACと総称）が70-80%、*M. kansasii* が20%を占める。リンパ節炎はMACや*M. scrofulaceum*、皮膚感染症は *M. marinum*、*M. fortuitum*、*M. chelonae*、*M. abscessus* や *M. ulcerans*（Buruli潰瘍）、播種性感染症はMAC、*M. kansasii*、*M. chelonae*、*M. abscessus* や *M. haemophilum* などが多い。NTMにBSL-3病原体はなく、ほとんどのNTMはBSL-2に分類されている。

NTM感染症の好発要因として、進行したAIDS（末梢血CD4陽性T細胞数≤100/μl）など免疫不全、肺基礎疾患（気管支拡張症、肺嚢胞、塵肺や陳旧

性結核など)、抗 interferon-γ自己抗体やサイトカイン(腫瘍壊死因子-αなど)阻害療法が知られている。しかし、既知の好発要因を欠如した症例(中年以降の女性)もしばしば認められる。MAC は多くの抗結核薬に耐性を示し、比較的有効なマクロライド系抗菌薬(clarithromycin や azithromycin)が治療に用いられているが、根治は困難である。なお、*M. kansasii* は通常の抗結核薬に感受性を示すことが多い。

表 2. 非結核性抗酸菌(NTM)感染症の概要

発生動向:	● 結核を含む抗酸菌感染症の約 10-20%を占める(世界:100-200 万人、日本:2,500-5,000 人/年) ● 結核低蔓延国(アメリカ合衆国)では NTM 感染症 > 結核 ● NTM の菌種で *Mycobacterium avium* complex (MAC) が最頻 ● 進行した HIV 感染症(CD4 陽性 ≤ 100/μL)における日和見感染症として重要 ● ヒト-ヒト感染はほとんどなく、感染症法対象外
病態:	● MAC は環境菌であり、水、土壌や動物に普遍的に存在 ● 肉芽腫炎症、気管支拡張、播種性、病変部位では肺に多い ● MAC は抗微生物薬耐性であり、治療は難渋
診断:	● アメリカ合衆国胸部疾患学会・感染症学会の診断指針(2007)によるが、臨床(症状や画像所見)と微生物学的所見(培養)を加味するため、確定診断に少なくとも 1 ヶ月を要する ● 迅速・簡便・安全な診断方法の開発が希求されている

最近の話題

結核

結核菌の全自動遺伝子検出系

最近、迅速(100 分以内)、高感度・特異度で結核菌と rifampicin (RIF) 耐性結核菌を同時検出可能な全自動遺伝子検出系(Xpert MTB/RIF)が開発され、簡便な操作や安全性も確保され、その高い臨床的有用性が示された(5)。Xpert MTB/RIF は多剤耐性結核対策にも威力を発揮することが期待される(3)。

日本発新規抗結核薬

近年、新規抗結核薬の研究・開発が活発であり、特に、多剤耐性結核菌を標的とした adenosine triphosphate (ATP) 合成酵素阻害薬:bedaquiline (TMC207、Johnson & Johnson)やミコール酸合成阻害薬:delamanid (OPC-67683、大塚製薬)が注目されている。

結核菌は細胞壁に特徴的な糖脂質(trehalose dimycolate など)を含有している。糖脂質ミコール酸合成系を標的とした nitroimidazole 系新規抗結核化学療法薬(delamanid、大塚製薬)が開発された。最少発育阻止濃度(MIC)、有効性や安全性に優れ、ヒトにおける高い忍容性が認められている。多剤耐性結核を対象とした delamanid の多施設臨床試験結果は1)高い菌陰性化率および2)早期抗菌活性(投与後 8 週以内に 45%が菌陰性化)を示し、高い臨床的有用性が証明された(6)。Delamanid は欧州連合に既に承認申請中、日本においても申請準備中である。結核の治療期間(現行:6 ヶ月)の短縮や多剤耐性結核対策に貢献することが期待される。

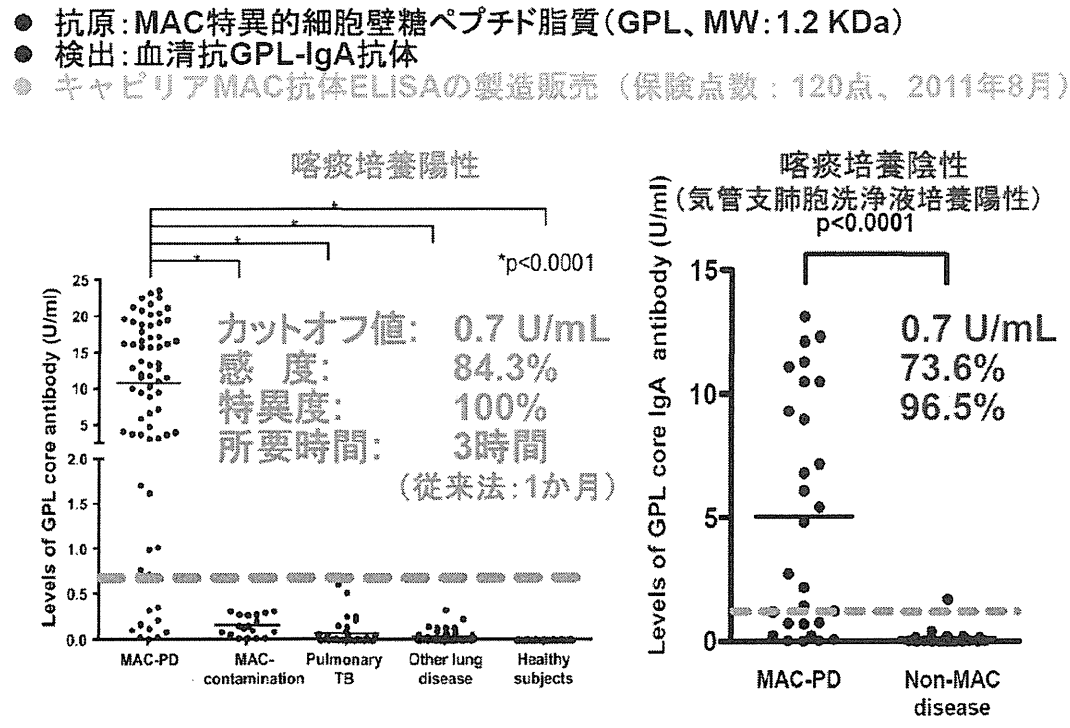
非結核性抗酸菌感染症

日本発 MAC 感染症の新規血清診断

MAC を含む NTM 症の診断はアメリカ合衆国胸部疾患学会・感染症学会の診断基準(2007)による(4)。その要点は、1. 臨床所見として1)呼吸器症状(咳嗽や喀痰)があり、2)画像所見(単純胸部X線で結節あるいは空洞病変が認められる、あるいは、高解像度コンピューター断層撮影(CT)による多発性小結節を伴う多巣性気管支拡張)を認め、および3)他疾患が除外され、2. 微生物学的所見として1)複数の喀痰検体で 2 回以上の培養陽性、2)気管支洗浄液で 1 回以上の培養陽性、3)経気管支内視鏡下あるいは開胸肺生検で肉芽腫炎症および 1 回以上の NTM 培養陽性のいずれかの条件を満たすことが基準となる。しかし、

確定診断に臨床と微生物所見を加味するため、複雑であり、かつ、確定診断に少なくとも1ヵ月を要すること、また、MACを含むNTM症は「感染症法」の対象外であるが、多くの肺MAC症患者は喀痰抗酸菌塗抹陽性の時点で最寄りの保健所に届けられ、ヒト—ヒト感染がないにも拘わらず、不要な隔離・治療を余儀なくされていることがあり、迅速かつ簡便な診断方法が望まれている。

MAC特異的細胞壁糖ペプチド脂質（glycopeptidolipid、GPL）は分子量約1.2 kDaの主要抗原である。化学的にGPLは全てのMAC（28血清型）に共通なGPL核と可変的な糖鎖部分から構成される。GPLは遅発育菌であるMACおよび*M. scrofulaceum*や迅速発育菌である*M. chelonae*および*M. fortuitum*はGPLを保有するが，結核菌群（BCGを含む）や*M. kansasii*は非保有である。GPL抗原特異性の観点から血清抗GPL-IgA抗体検出によるMAC感染症の迅速血清診断法（所要時間3時間）が開発された（7-10）。活動性MAC感染症の血清診断に関し、多施設共同研究の結果として、喀痰培養陽性MAC患者の血清診断はアメリカ合衆国胸部疾患学会・感染症学会の診断基準（2007）に比し、感度は84.3%，特異度は100%（9）であり，喀痰培養陰性MAC患者（気管支肺胞洗浄液培養陽性）の感度は73.6%，特異度は96.5%であった（10）（図2）。MAC-GPL抗原を用いた血清抗体検出は活動性MAC感染症の非侵襲性で安全な迅速診断法として有用である。GPL非保有抗酸菌感染症、通常の細菌性肺炎、肺がん、間質性肺炎、慢性閉塞性肺疾患、サルコイドーシス、健常者、さらに、診断基準を満足なMAC contamination/colonizationは陰性であり、血清診断はMAC感染症を特異的に検出し、迅速な鑑別診断にも優れている。また、抗体価は活動性＞非活動性MAC感染症であり、疾患活動性も反映した（7，8）。厚生労働省からMAC-GPL血清診断キットの体外診断用医薬品製造販売が承認され、2011年8月22日に保険収載、一般検査項目として臨床使用が開始されている（キャピリア®MAC抗体ELISA、タウンズ）。今後、MAC-GPL血清診断キットの海外における性能評価や市販後性能評価が進み、MAC-GPL血清抗体価が国際的診断基準に追加されることが望まれる。



Am. J. Respir. Crit. Care Med. 2008; 177: 793-797. Chest 2010; 138: 236-237.

図2．活動性肺MAC感染症の血清診断

結　語

抗酸菌感染症には結核、非結核性抗酸菌（NTM）感染症やハンセン病(Hansen's disease、leprosy)などがあり、現在でも、多くの抗酸菌感染症患者が存在し、人類に甚大な健康被害を与えている。結核など抗酸菌感染症の動向や最近の革新的な話題（全自動結核菌遺伝子検出系、新規抗結核薬であるdelamanid、MAC感染症の血清診断）について概説した。特に、delamanidやMAC感染症の血清診断は日本発であり、日本の科学技術やその応用により、世界の抗酸菌感染症対策に寄与することが期待される。

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本論文は発表内容関連して他者との利害関係なく、特に申告事項はない。

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Major T Cell Response to a Mycolyl Glycolipid Is Mediated by CD1c Molecules in Rhesus Macaques

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Human CD1b molecules contain a maze of hydrophobic pockets and a tunnel capable of accommodating the unusually long, branched acyl chain of mycolic acids, an essential fatty acid component of the cell wall of mycobacteria. It has been accepted that CD1b-bound mycolic acids constitute a scaffold for mycolate-containing (glyco)lipids stimulating CD1b-restricted T cells. Remarkable homology in amino acid sequence is observed between human and monkey CD1b molecules, and indeed, monkey CD1b molecules are able to bind glucose monomycolate (GMM), a glucosylated species of mycolic acids, and present it to specific human T cells *in vitro*. Nevertheless, we found, unexpectedly, that *Mycobacterium bovis* bacillus Calmette-Guerin (BCG)-vaccinated monkeys exhibited GMM-specific T cell responses that were restricted by CD1c rather than CD1b molecules. GMM-specific, CD1c-restricted T cells were detected in the circulation of all 4 rhesus macaque monkeys tested after but not before vaccination with BCG. The circulating GMM-specific T cells were detected broadly in both $CD4^+$ and $CD8^+$ cell populations, and upon antigenic stimulation, a majority of the GMM-specific T cells produced both gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α), two major host protective cytokines functioning against infection with mycobacteria. Furthermore, the GMM-specific T cells were able to extravasate and approach the site of infection where $CD1c^+$ cells accumulated. These observations indicate a previously inconceivable role for primate CD1c molecules in eliciting T cell responses to mycolate-containing antigens.

Group 1 CD1 molecules bind a variety of lipidic antigens (Ags) and present them to specific T cells. In humans, three group 1 CD1 molecules, namely, CD1a, -b, and -c, exist that have evolved mutually distinct Ag-binding grooves. Therefore, a group of microbial Ags with unique lipid tails may bind preferentially to a particular CD1 isoform. The lipid species essential for the cell wall architecture of mycobacteria include a family of α-alkyl-β-hydroxy fatty acids with an extremely long acyl chain, termed mycolic acids, which Beckman and colleagues identified as a CD1b-presented Ag (1). Subsequently, glucose monomycolate (GMM), a glucosylated species of mycolic acid, was also shown to be presented by human CD1b molecules (2), and the crystal structure of the GMM-CD1b complex underscored that the acyl chain of GMM fitted tightly in a maze of pockets and a tunnel elaborated in human CD1b molecules (3). Furthermore, glycerol monomycolate can also be presented by human CD1b molecules, leading to the assumption that CD1b-bound mycolic acids constitute a scaffold for mycolate-containing (glyco)lipids stimulating CD1b-restricted T cells (4). A potential link between GMM and glycerol monomycolate and the active and latent phases of human tuberculosis, respectively, has been proposed (4, 5), and studies of immune responses to these Ags in experimental animals are now important for future advances in this field.

Mice and rats have lost all the group 1 CD1 genes, and the reconstitution of human group 1 functions in mice by gene transfer has provided significant insights (6); however, it is unclear whether the CD1-restricted T cell response generated in transgenic mice faithfully represents that naturally elicited in humans. Alternatively, animals, such as guinea pigs (7) and cows (8), that are naturally equipped with the group 1 CD1 system have been utilized, but the number and the expression patterns of the group 1 CD1 isoforms differ significantly from those in humans. Obviously, a fair prediction would be that nonhuman primates will serve as reliable animal models, and indeed, our previous work has indicated that the group 1 CD1 system is highly conserved between humans and rhesus macaque monkeys (9). Furthermore, monkey CD1b molecules were capable of binding GMM and presenting it to T cells expressing GMM-specific, human CD1b-restricted T cell receptors (9).

To extend this work further in an *in vivo* system, the current study was initially designed to monitor GMM-specific T cell responses in *Mycobacterium bovis* bacillus Calmette-Guerin (BCG)-immunized monkeys. During the course of the study, we found that a major T cell response to GMM in these animals was restricted by CD1c molecules. Upon antigenic stimulation, the GMM-specific T cells produced host protective cytokines. Furthermore, GMM-specific T cells were recruited to the site of infection where $CD1c^+$ cells aggregated, suggesting their role in host defense against mycobacterial infections.

MATERIALS AND METHODS

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 Nonhuman Primates at the Institute for Virus

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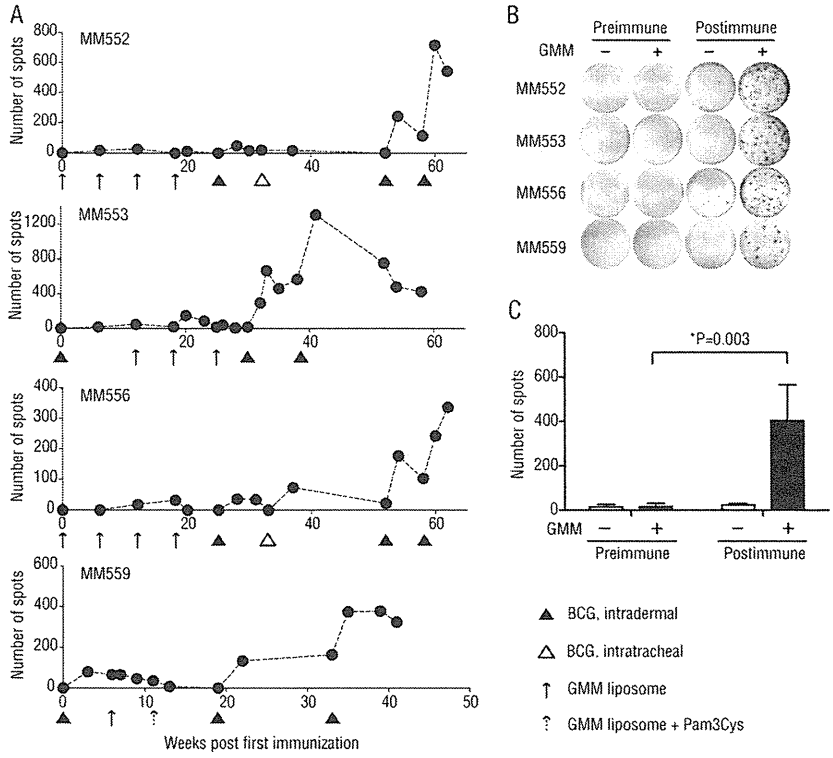

FIG 1 GMM-specific T cell responses induced in BCG-immunized monkeys. (A) Four monkeys were vaccinated intradermally with BCG and GMM liposome. In some cases, BCG was administered via an intratracheal route and the GMM liposome was injected along with the Pam3Cys-SKKKK adjuvant. At the indicated time points, PBMCs were obtained, and the number of GMM-specific cells was determined by IFN-γ ELISPOT assays. The numbers of the GMM-specific spots per 1×10^6 PBMCs are plotted. (B and C) Preimmune PBMCs and those obtained after the third BCG immunization were incubated with either the GMM liposome (GMM +) or empty liposome (GMM −), followed by detection of IFN-γ-producing cells in an ELISPOT assay. Statistical analysis was performed using a one-way analysis of variance (ANOVA). Error bars show standard deviations.

Research, Kyoto University. The Tokyo 172 strain of BCG (Japan BCG Laboratory, Tokyo, Japan) was grown in 7H9 medium and harvested at its mid-log-phase growth as described previously (7). GMM was purified from cultured mycobacteria and integrated into stearylated octaarginine-containing liposomes as described previously (7). Four rhesus macaque monkeys were vaccinated intradermally with 1×10^8 CFU of BCG and challenged with 50 μg of the GMM liposome at various intervals from 6 to 20 weeks as shown in Figure 1A. The dose of BCG was determined on the basis of our previous studies with guinea pigs (7). In some cases, BCG was administered via an intratracheal route using a bronchoscope, and the GMM liposome was injected along with 100 μg of Pam_3Cys-SKKKK (EMC Microcollections, Tuebingen, Germany).

IFN-γ ELISPOT assays. Gamma interferon (IFN-γ) enzyme-linked immunosorbent spot (ELISPOT) assays were performed using a human/monkey IFN-γ ELISPOT kit (Mabtech, Nacka Strand, Sweden) as described previously (10), with slight modifications. In blocking experiments, cells were preincubated with 5 μg/ml of anti-CD1a (10H3), CD1b (b3.1), CD1c (M241), or negative control (P3) antibodies (Abs) for 20 min before Ag exposure. In some experiments, responder T cells were purified from peripheral blood mononuclear cells (PBMCs) by negative selection with anti-CD1c (M241), CD14 (M5E2), CD16 (3G8), CD20 (2H7), and CD56 (B159) Abs, and ELISPOT assays were performed using the LLC-MK2 rhesus macaque kidney epithelial cell line expressing rhesus macaque CD1a, CD1b, or CD1c (9) as Ag-presenting cells (APCs).

Derivation of T cell lines and T cell assays. GMM-specific T cell lines were established independently from MM552 and MM553. Peripheral blood mononuclear cells (PBMCs) (1.1×10^7/well) were cultured with the GMM liposome (1 μg/ml), and antigenic stimulation was repeated every 2 weeks in the presence of irradiated autologous PBMCs. The culture medium used was RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal calf serum (HyClone, Logan, UT), 3 nM interleukin-2 (IL-2), 2-mercaptoethanol (Invitrogen, Carlsbad, CA), penicillin, and streptomycin. For T cell assays, the cells (5×10^4/well) were incubated with the GMM liposome (1 μg/ml) in the presence of LLC-MK2 cell transfectants (5×10^4/well) expressing rhesus macaque CD1a, CD1b, or CD1c using a 96-well flat-bottom microtiter plate. After 24 h, aliquots of the culture supernatants were collected, and the amount of IFN-γ released into the medium was measured using a human/monkey IFN-γ enzyme-linked immunosorbent assay (ELISA) kit (Mabtech).

Flow cytometry. Freshly isolated PBMCs (5×10^5/well) were stimulated with either empty liposome or the GMM liposome (1 μg/ml) for 6 h in 96-well flat plates and then treated with brefeldin A for an additional 6 h. Subsequently, the cells were harvested and labeled with anti-CD8 Ab (RPA-T8; phycoerythrin [PE]-Cy7) and anti-CD4 Ab (OKT4; eFluor 450). This was followed by cell fixation and permeabilization. The cells were then labeled with anti-IFN-γ Ab (4S.B3; PE) and anti-tumor necrosis factor alpha (TNF-α) Ab (monoclonal antibody 11 [MAb11]; fluorescein isothiocyanate [FITC]) and analyzed by flow cytometry using a BD FACS CantoII flow cytometer (BD Biosciences).

Adoptive transfer. Cells (1×10^7) of the MM552-derived, GMM-specific T cell line were labeled with 5 μM carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen) for 10 min at 37°C. The labeled cells were suspended in 10 ml of glucose lactated Ringer's solution and injected intravenously into the MM552 monkey from which the T cells were derived. At the same time, the MM552 monkey received an intradermal inoculation with BCG (1×10^8 CFU). After 4 days, the monkey was sacrificed, and skin samples were collected. The excised skin was fixed

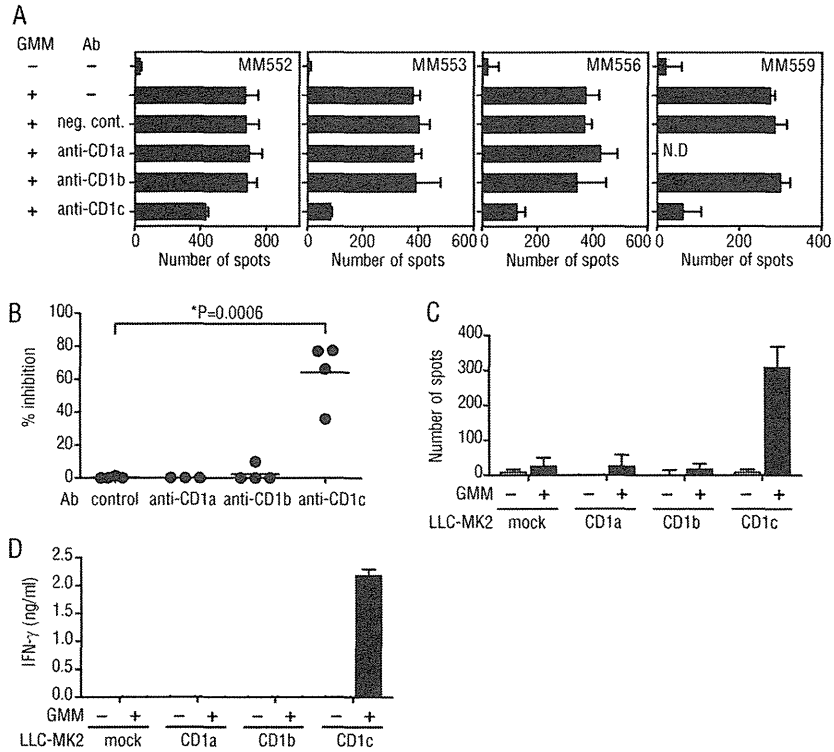

FIG 2 The GMM-specific T cell response was dependent on CD1c molecules. (A and B) Postimmune PBMCs from the monkeys were subjected to IFN-γ ELISPOT assays in the presence or absence of anti-CD1 MAbs, and the numbers of GMM-specific spots per 1×10^6 PBMCs are shown (A). The percentage of inhibition by anti-CD1 Abs in each monkey was plotted, and statistical analysis was performed using a one-way ANOVA (B). (C) Postimmune PBMCs were isolated from MM553, and negative selection with Abs to CD1c, CD14, CD16, CD20, and CD56 was performed. Subsequently, the cells were subjected to a IFN-γ ELISPOT assay using LLC-MK2 cell transfectants expressing monkey CD1a, CD1b, or CD1c molecules as APCs. (D) Cells of the GMM-specific monkey T cell line established from MM553 were incubated with the LLC-MK2 cell transfectants in the presence or absence of the GMM liposome, and the amount of IFN-γ released into the medium was measured by a IFN-γ ELISA. neg. cont., negative control. Error bars show standard deviations.

with 4% paraformaldehyde and deep-frozen in optimal cutting temperature (OCT) compound, and the cryosections were mounted with Vectashield mounting medium with DAPI (4′,6′-diamidino-2-phenylindole; Vector Laboratories) and viewed under a fluorescence microscope. Some cryosections were processed for immunohistochemistry as described previously (11) and labeled with M241 or P3. Signals were visualized with the standard protocol using a Vector Elite ABC kit.

RESULTS

GMM-specific T cell responses were induced in BCG-vaccinated monkeys. We previously detected GMM-specific T cell responses in BCG-vaccinated guinea pigs (7). To see if such T cell responses were also induced in nonhuman primates, four rhesus macaque monkeys (MM552, MM553, MM556, and MM559) received intradermal inoculation with BCG 3 times during the course of the study (Fig. 1A), and MM552 and MM556 were additionally challenged with BCG via an intratracheal route. We also attempted to sensitize the animals by intradermal injection of the GMM liposome either with or without the Pam3Cys-SKKKK lipopeptide adjuvant. At the time points indicated in Figure 1A, PBMCs were obtained from the monkeys and tested for their ability to respond to GMM in IFN-γ ELISPOT assays. The GMM-specific response was only marginal after the first intradermal vaccination with BCG in all cases, but the response was more prominent after the second and the third vaccinations. We did not see such a robust response elicited by the administration of BCG via an intratracheal route (MM552 and MM556). Furthermore, no implications were obtained for the potential of the GMM liposome in terms of inducing GMM-specific T cell responses (Fig. 1A).

Preimmune PBMCs and those after the third BCG vaccination were obtained from each subject, and a IFN-γ ELISPOT assay was performed in parallel (Fig. 1B). Numerous spots were detected when PBMCs obtained after the third BCG vaccination were stimulated *in vitro* with the GMM liposom, but not with empty liposome, whereas preimmune PBMCs failed to respond to GMM (Fig. 1B, postimmune, + and −, and preimmune, +, respectively). The increase in the number of GMM-specific spots after BCG vaccination was statistically significant (Fig. 1C). Therefore, as in guinea pigs, GMM-specific T cell responses were elicited by BCG vaccination in rhesus macaque monkeys.

The GMM-specific response was restricted by CD1c molecules. Given that monkey CD1b molecules present GMM to specific T cells *in vitro* (9), we initially predicted that CD1b molecules would function as the restriction element for the GMM-specific response induced *in vivo* by BCG vaccination. However, blocking studies with a panel of anti-CD1a (10H3), anti-CD1b (b3.1), and anti-CD1c (M241) MAbs that were known to specifically block human T cell responses and cross-react to the corresponding monkey CD1 isoforms (9) indicated that CD1c rather than CD1b molecules restricted the GMM-specific response in all 4 monkeys (Fig. 2A). The inhibitory effect of anti-CD1c but not anti-CD1b Abs was statistically significant (Fig. 2B). To confirm that the GMM-specific T cell response was restricted by CD1c molecules,

peripheral blood T cells were purified from a BCG-vaccinated monkey and tested for their ability to respond to GMM when LLC-MK2 cell transfectants expressing rhesus macaque CD1a, CD1b, or CD1c molecules were used as APCs. In a IFN-γ ELISPOT assay, significant numbers of GMM-specific T cell spots were detected only in the presence of $CD1c^+$ LLC-MK2 cells (Fig. 2C). Finally, a GMM-specific T cell line established from the MM553 monkey responded to GMM by producing IFN-γ when LLC-MK2 cell transfectants expressing CD1c but not those expressing the other CD1 isoforms were used as APCs (Fig. 2D). Taken together, these lines of evidence indicate an outstanding ability of rhesus macaque CD1c molecules to mediate GMM-specific T cell responses.

GMM-specific T cells were detected broadly in $CD4^+$ and $CD8^+$ T cell populations. Initial studies of group 1 CD1-restricted T cell lines (1, 12), as well as our previous study of BCG-vaccinated humans (13), suggested that group 1 CD1-restricted T cells were enriched in the $CD4^-$ T cell population. However, recent evidence obtained from human cases with tuberculosis indicated that a majority of GMM-specific, CD1b-restricted T cells was $CD4^+$ (14). To determine the phenotype of GMM-specific T cells detected in our monkey model, PBMCs derived from the 4 BCG-vaccinated monkeys were stimulated *in vitro* with either the GMM liposome or empty liposome and subjected to a multicolor flow cytometric analysis for intracellular cytokines and surface markers. In all cases, a T cell population expressing both IFN-γ and TNF-α was readily detectable when cells were stimulated with the GMM liposome (Fig. 3; boxed in bold lines). The GMM-reactive T cell population thus identified was further separated, based on the cell surface expression of CD4 and CD8α glycoproteins, into CD4 single-positive (SP), CD8 SP, and CD4 CD8 double-positive (DP) cells (Fig. 3). We found that the GMM-reactive T cells were detected broadly in CD4 SP, CD8 SP, and DP populations except for MM553, in which the GMM-reactive T cells were detected predominantly in CD4 SP and DP populations. It was not surprising that Ag-specific DP T cells were detected in this study because such T cells are known to comprise a significant population of memory T cells in the peripheral blood of monkeys (15). Upon antigenic stimulation, the GMM-reactive T cells did not upregulate transcription of IL-17A and IL-17F (data not shown), suggesting that these T cells were distinct from Th17 cells.

GMM-specific T cells were recruited to the site of infection. Circulating GMM-specific T cells are detectable in patients with tuberculosis (14), but it remains to be examined if these cells are able to extravasate and gain access to the site of infection. To address this, the GMM-specific T cell line derived from the MM552 monkey was labeled with CFSE and injected intravenously back into the same subject, i.e., MM552. At the time of injection with CFSE-labeled cells, BCG was inoculated in the skin, and after 4 days, the BCG-infected skin was examined for infiltration by the CFSE-labeled cells. The local accumulation of $CD1c^+$ cells was prominent in the infected tissue (Fig. 4A, bottom right), and CFSE-labeled T cells (Fig. 4B, bottom left, indicated with arrowheads) penetrated deeply into the granulomatous cell aggregates formed at the site of BCG infection. Such cellular responses were undetectable in uninfected skin areas (Fig. 4A and B, top). Taken together, these observations indicated that, in response to BCG vaccination, GMM-specific, CD1c-restricted T cells could expand in the circulation with the potential for mobilization to the site of mycobacterial infection.

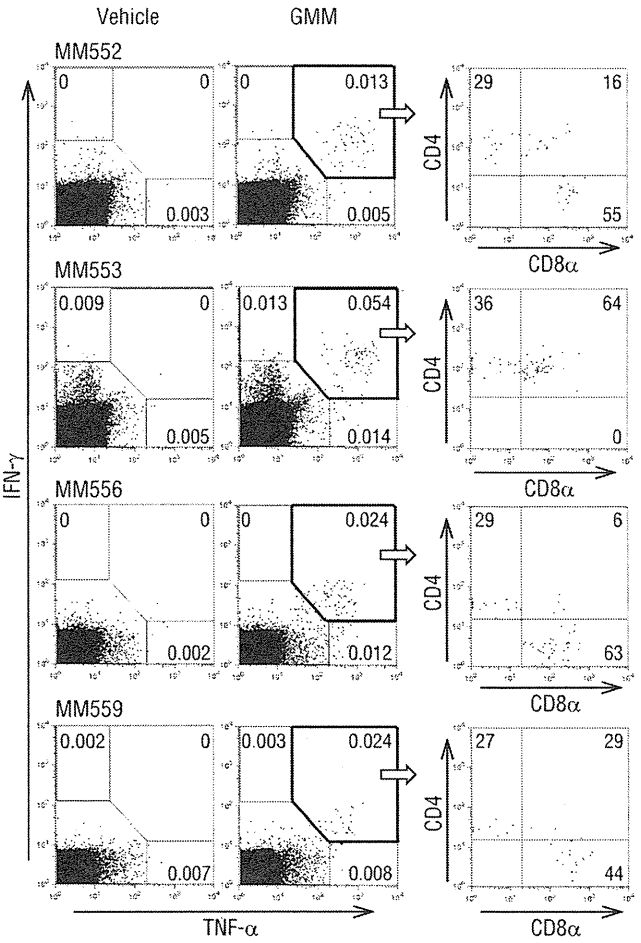

FIG 3 Profiles for surface marker expression on monkey GMM-specific T cells. PBMCs obtained from the BCG-vaccinated monkeys were incubated for 6 h with either empty liposome or the GMM liposome, and then brefeldin A was added to the culture. After an additional 6-h incubation, the cells were fixed, permeabilized, and labeled with Abs to CD4, CD8, IFN-γ, and TNF-α. This was followed by a multicolor flow cytometric analysis. The IFN-γ^+ TNF-α^+ cell population (boxed in bold lines) was further separated based on the expression of CD4 and CD8α molecules. The percentage of cells present in indicated regions is shown for each panel.

DISCUSSION

The present study provided evidence that monkey CD1c molecules were capable of functioning as a restriction element for GMM-specific T cells *in vitro* and eliciting GMM-specific T cell responses *in vivo* (Fig. 1). The crystal structure of the human CD1b-GMM complex indicated that the T′ tunnel connecting the A′ and F′ pockets is essential for accommodating the long meromycolate chain (3). The tunnel structure constructed in human CD1b molecules is obstructed in human CD1a and CD1c isoforms by replacement of the glycine residue at position 98 (CD1b) with valine (CD1a and CD1c). Although the crystal structure has not yet been elucidated, monkey CD1c molecules exhibit much higher homology with human CD1c molecules (90.4% in the amino acid sequence) than with human CD1b (58.9%) and have valine at position 98, making it unlikely that the meromycolate chain-accommodating T′ tunnel detected for human CD1b is constructed in monkey CD1c. The crystal structure of human CD1c molecules was resolved recently, indicating significant dif-

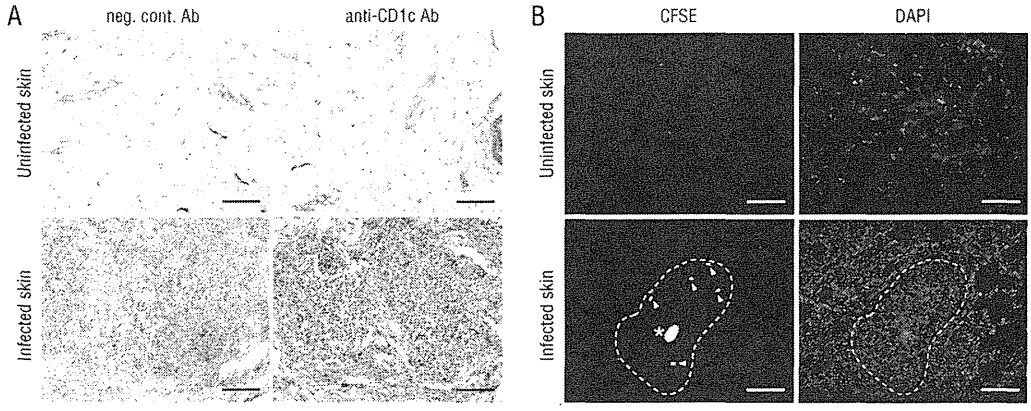

FIG 4 Recruitment of GMM-specific T cells to the site of infection. The CFSE-labeled, GMM-specific T cells were injected into the circulation of the donor. At the same time, BCG (1×10^8 CFU) was inoculated into the skin. After 4 days, samples of the infected (bottom, BCG-positive) or uninfected (top, BCG-negative) skin were obtained and examined for infiltration by $CD1c^+$ cells (A) and CFSE-labeled cells (B). The tissue sections were also counterstained with DAPI (B). Arrowheads indicate CFSE-positive cells, and dashed lines indicate the area of granulomatous macrophage aggregation. The amorphous fluorescence-positive structure (indicated with an asterisk) appeared to represent the necrotic center of the granuloma. neg. cont., negative control. Scale bars, 100 μm.

ferences between human CD1b and CD1c molecules (16). The human CD1c molecule contains two deep grooves, each of which has a portal (D′ portal and E′ portal) at the bottom. As the two portals are located in close proximity, one potential mechanism for interaction between monkey CD1c and the long meromycolate chain of GMM is that the fatty acyl could exit one portal and immediately enter the other, minimizing its uncomfortable exposure to the aqueous external milieu. The crystallographic structure of monkey CD1c molecules is under investigation and should provide valuable insights into how monkey and, possibly, human CD1c molecules bind GMM.

In the present study, we performed an adoptive transfer experiment, which is a common approach in studies with genetically identical experimental rodents but is challenging in nonhuman primates. This is probably the first direct demonstration that group 1 CD1-restricted T cells are recruited from the circulation to the site of infection (Fig. 4). These T cells produce IFN-γ and TNF-α, representative cytokines critical for host defense against mycobacterial infections (Fig. 3). Furthermore, inoculation of purified GMM into the skin of BCG-vaccinated monkeys resulted in elicitation of delayed-type hypersensitivity (DTH), as in guinea pigs (7), that was associated with the expression of microbicidal agents, such as granulysin (data not shown). Given that GMM is produced in tissues by utilizing host-derived glucose as a substrate for mycolyltransferases (5), GMM-specific T cells may be particularly important for eliminating metabolically active, replicating microbes.

Monkey studies are often recognized as a surrogate for human studies. On the other hand, monkey studies may occasionally provide compelling new insights that have never been noted in a full range of human studies. Despite significant advances in the biology of group 1 CD1 molecules over the past 2 decades, the GMM-specific, CD1c-restricted T cell response in monkeys was unexpected and is stimulating enough to address whether such responses may exist in humans and how CD1b and CD1c play distinct roles.

ACKNOWLEDGMENTS

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Title:

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

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Running title:

Immunogenicity of DosR regulon-encoded proteins

Keywords:

Mycobacterium tuberculosis, vaccine, dormancy, DosR regulon

SUMMARY

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

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

DESIGN: T-cell responses against 33DosR regulon-encoded antigens were investigated in 26 consecutive *Mtb*-infected individuals comprising 14 individuals with latent TB infection (LTBI) and 12 patients with active pulmonary TB (ATB) using Enzyme-linked immunosorbent spot assay, and antibody responses in 42 consecutive individuals (14 LTBI and 28 ATB) using Enzyme-linked immunosorbent assay.

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

CONCLUSION: T-cell responses to six antigens might contribute to natural protection against dormant *Mtb*. Therefore, these antigens are considered to be potential targets of novel vaccines for the Japanese population to control *Mtb* reactivation.

INTRODUCTION

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

In individuals with latent TB infection (LTBI), *Mtb* bacilli persist within the human body, especially in the lung granuloma, and can survive for long time periods in a reversible metabolically inactive state. Some LTBI develop active TB by reactivation of *Mtb* if the host's cell-mediated immune response, which plays a pivotal role in protection against TB, is impaired by some cause (aging, HIV infection, immunosuppressive treatment, etc.). This reactivation of persistent *Mtb* bacilli is the major cause of adult pulmonary TB.[2-4]

At present, *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) is the only available prophylactic vaccine against *Mtb*. BCG vaccination to newborns and infants is an effective preventive measure against initial active TB disease, whereas its prophylactic efficacy in adults is limited.[5-7] Various anti-TB vaccine forms such as DNA vaccines, recombinant BCG vaccines, subunit vaccines, and attenuated TB vaccines are under development, but they have not so far achieved an effect superior to BCG .[5-7] Because adult TB is largely due to the reactivation of dormant *Mtb*, 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 *Mtb* dormancy, are up-regulated by some stresses such as hypoxia, low pH, nitric oxide, and carbon monoxide.[8-10] Such evidence suggests that DosR regulon plays an essential role in adaptation to these stressed conditions, although the detailed functions of most proteins encoded within this regulon are not fully understood. These DosR regulon-encoded proteins are also demonstrated to have immunogenicity in *Mtb*-infected individuals,[11-16] suggesting that they are suitable targets for vaccines to control the reactivation of dormant *Mtb*.

Immune responses against *Mtb* have been preferentially measured by interferon (IFN)-γ release assays (IGRA), because IFN-γ produced by $CD4^+$ helper T-cells (Th1 cells) and $CD8^+$ cytotoxic T-cells (CTLs) plays a central role in the protection against *Mtb*.[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 the ethnic differences, bacterial prevalence, BCG vaccination history, and detection methods, as well as genetic differences in local pathogens.

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

MATERIALS AND METHODS

Study subjects

This prospective study was approved by the institutional review boards of Hamamatsu University School of Medicine and conducted according to the principles expressed in the Declaration of Helsinki. Only adults (≥20 years old) who had given written informed consent were included in this study. Twenty-six consecutive Japanese individuals comprising 14 individuals with latent TB infection (LTBI) and 12 patients with active pulmonary TB (ATB) were recruited to investigate T-cell responses against DosR regulon-encoded antigens (Table 1). In addition, 11 healthy controls (HC) were recruited to check the T-cell responses against 6 kDa early secretory antigenic target (ESAT-6) and 10 kDa culture filtrate antigen (CFP-10) together with those in LTBI and ATB. For serum antibody responses against DosR regulon-encoded antigens, 16 additional ATB subjects were also examined (Table 2). Consequently, 42 consecutive individuals comprising 14 LTBI and 28 ATB were recruited for antibody responses. All LTBI were asymptomatic volunteers or non-TB patients positive for the QuantiFERON®-TB Gold In-Tube (QFT) test (Cellestis, Victoria, Australia). All LTBI had no evidence of active TB on clinical examination, chest radiography, and microbiological examinations on sputum and/or bronchial lavage. ATB were thus excluded from LTBI, and no LTBI had developed active TB until date. The diagnosis of ATB by expert physicians was confirmed by Ziehl-Neelsen staining of sputum smears and bacterial culture studies for *Mtb*. Individuals having infectious diseases other than TB, immunosuppressive therapy, chemotherapy, autoimmune or hematologic diseases, or human immunodeficiency virus (HIV) infection were excluded in this study. All HC were negative for QFT and had no history of close TB exposure, anti-TB treatment, and prior active TB. Their chest radiography did not show any abnormal findings, and they

did not have any respiratory or systemic symptoms. In the present study, all HC had ever received BCG-vaccination.

Preparation of recombinant proteins

DNA encoding DosR regulon proteins was amplified by PCR using genomic DNA from *Mtb* H37Rv or BCG as a 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, 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) using a dye primer cycle sequencing kit (Applied Biosystems). The pET-23b(-) vectors containing ESAT-6 or CFP-10 genes were kindly provided by Drs. J.T. Belisle and K.M. Dobos (Department of Microbiology, Immunology, and Pathology, Colorado State University).

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, Ltd., Osaka, Japan). Proteins were extracted from the bacterial cells with 8M urea (Wako Pure Chemical Industries, Ltd.) 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). Purity and size of recombinant proteins were checked by SDS-PAGE. Residual endotoxin levels were determined by Endospecy ES-24S Kit and Toxicolor DIA Kit (Seikagaku Biobusiness Corporation, Tokyo, Japan), and were found to be below 50 IU/mg recombinant protein.

Enzyme-linked immunosorbent spot (ELISPOT) assay

In ATB, blood samples were obtained within two weeks of initiation of treatment. Peripheral blood mononuclear cells (PBMCs) were isolated from fresh venous blood by Ficoll-Paque PREMIUM (GE Healthcare). ELISPOT assay was performed with an antibody pair, horseradish peroxidase (HRP)-conjugated streptavidin, and 3-amino-9-ethylcarbazole (AEC) chromogen (Human IFN-γ ELISPOT pair,