り、さらに感染病変局所に最も多く集積している細胞もマクロファージであることから、マクロファージは結核菌感染に対する宿主防御反応である肉芽腫炎症において、中心的かつ必須の役割を演じている[3) 18)]。実際、in vitro肉芽腫炎症モデルを解析した結果、マクロファージおよびマクロファージ由来サイトカインが肉芽腫の最小構成単位を形成していることが判明し、in vivo病変形成機序を支持している[15)]。

マクロファージ抑制物質（IL-4，プロスタグランディンE、糖質コルチコイド）やIL-1受容体拮抗蛋白（IL-1ra）は、炎症性サイトカイン発現や活性を抑制する作用があり、肉芽腫炎症抑制物質である[19)]。反応性酸素化合物（ROIs）は単球／マクロファージ由来IL-1の産生誘導活性を有し、スーパーオキシドジスムターゼ（SOD）投与により肉芽腫炎症は抑制される[20)]。したがって、ROIsは肉芽腫炎症惹起物質であり、SODは肉芽腫炎症抑制物質である。

肉芽腫炎症と感染防御の統御

結核菌など抗酸菌は細胞内寄生病原体であり、宿主防御にマクロファージ-サイトカイン-CD4陽性1型ヘルパーT（Th1）細胞応答系、細胞性免疫が貢献している（**図2**）。

細胞性免疫の起動サイトカインとして、IL-12，IL-18やインターフェロン（IFN）-γがTh1細胞分化や活性化など、重要な役割を演じている[21)]。しかし、結核菌感染に対する遅延型過敏反応を含む細胞性免疫の発現は抗結核菌防御と組織傷害に貢献、すなわち功罪の二面性（諸刃の剣）を表現する[18)]。

また遺伝的因子として、ヒト第2染色体に存在する遺伝子（*NRAMP1*；natural resistance associated macrophage protein 1，別名*SLC11A1*）が感染防御に関与し、この機能はマクロファージに表現されている[22) 23)]。抗酸菌感染部位における宿主応答はマクロファージ（類上皮細胞や多核巨細胞を含む）およびT細胞の局所集積を特徴とする肉芽腫炎症であり、その成立機序には細胞性免疫発現が関与している[3) 18) 24)]。肉芽腫炎症は遺伝的感染感受性を示す宿主に顕著である[3) 9) 25)～27)]。

すなわち、*Nramp1*遺伝子感受性を表現するマウスでは抗酸菌増殖および肉芽腫炎症増強、炎症性サイトカイン（IL-1，TNF-αや単球走化性ケモカインなど）産生亢進、防御性サイトカイン（IL-12やIL-18）産生低下が判明し、抵抗性マウスではその逆であっ

図1　結核性肉芽腫炎症の細胞・機能分子機序

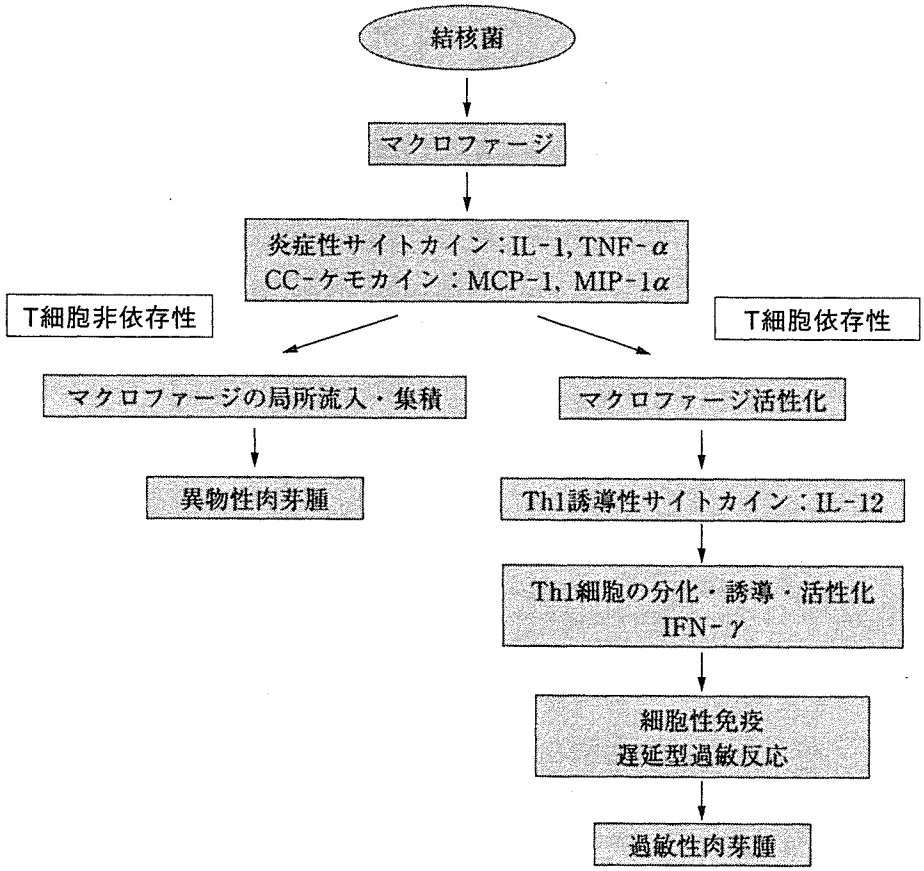
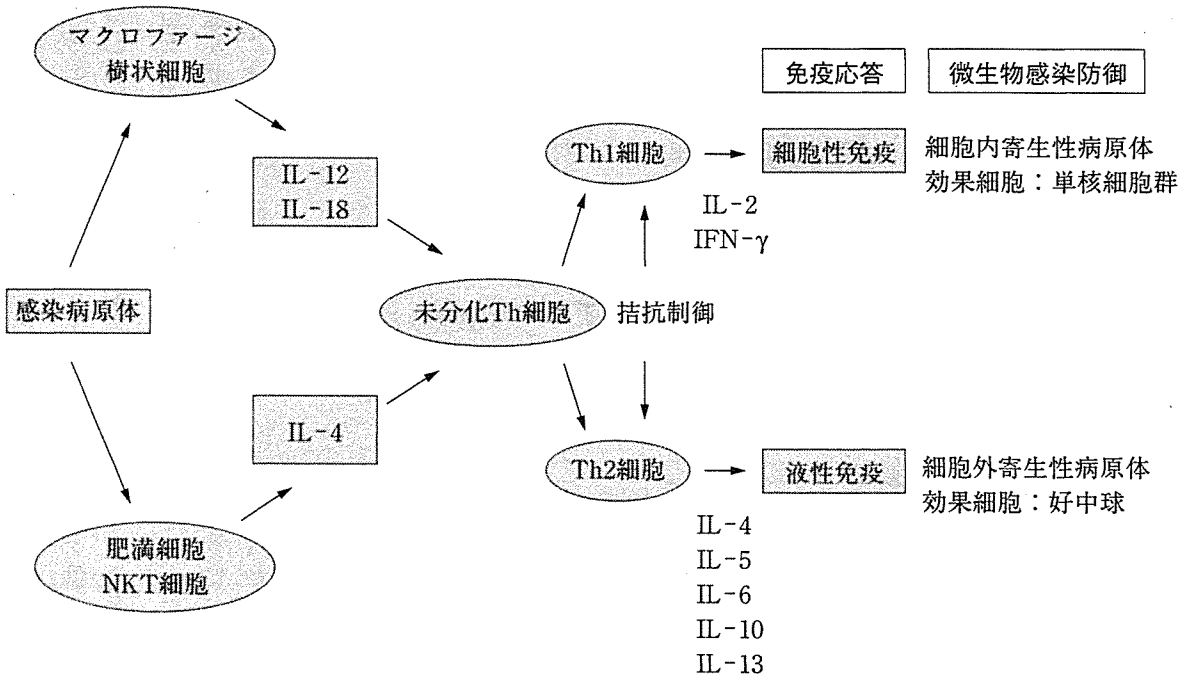


図2　微生物感染における細胞性および液性免疫応答



た。肉芽腫炎症は結核菌など宿主に侵入・感染した抗酸菌を局所に封じ込め、かつ、結核性肉芽腫の特徴である乾酪壊死は無血管領域であるため、好気性である結核菌の発育・増殖を抑制し、宿主に合目的な防御応答である。

これらの事実は、抗酸菌に対する宿主防御応答である肉芽腫炎症は、遺伝的抵抗性宿主において肉芽腫炎症を発現しなくても抗酸菌感染を制圧することが可能であり、感染防御にこの病変形成は不要であるが、感受性宿主には生存や防御のため必須であることを示している(図3)。

結核菌感染における宿主応答と細胞壁糖脂質

結核菌の脂質は乾燥菌体重量の一〇%以上、細胞壁の二〇%以上を構成し、他の一般細菌に比し、きわめて多い。事実、結核菌の全ゲノムは約四・四Mb(大腸菌四・六Mb)であり、蛋白質を規定している遺伝子は約四〇〇〇、脂肪酸代謝に関与している酵素は二五〇以上、大腸菌が五〇であることから、結核菌の脂質代謝がきわめて旺盛であることが遺伝子情報からも判明している[27]。

結核菌細胞壁の脂質として、mycolyl-arabinogalactan-peptidoglycan complex, lipoarabinomannan (LAM), lipomannan, phosphatidyl-myo-inositol, sulfolipid (SL), trehalose 6, 6'-dimycolate (TDM) /cord factor, phenolic glycolipid や lipooligosaccharides などの糖脂質が特徴的である[28]。

特に、アシル化trehalose脂質化合物であるTDM/cord factorやSLが結核菌に特徴的であり、結核菌-宿主関係、すなわち、病原性や毒性の発現に関与している。また、〝抗酸性〟に主として関与する菌体表層成分はミコール酸などの脂質成分であり、ミコール酸は天然で稀なα位に分枝鎖、β位に水酸基を持つ長鎖脂肪酸(結核菌では炭素数六〇～九〇)である。

宿主マクロファージは吸入した結核菌を貪食し、食胞体(phago-

some)を形成する。アシル化trehalose脂質化合物(SLやTDM)は食胞体と加水分解酵素を含むリソソーム(lysosome)の融合(P-L fusion)を阻止することにより、結核菌が酸性化されず、食胞体内、すなわち宿主マクロファージ内で生存を可能にしている。

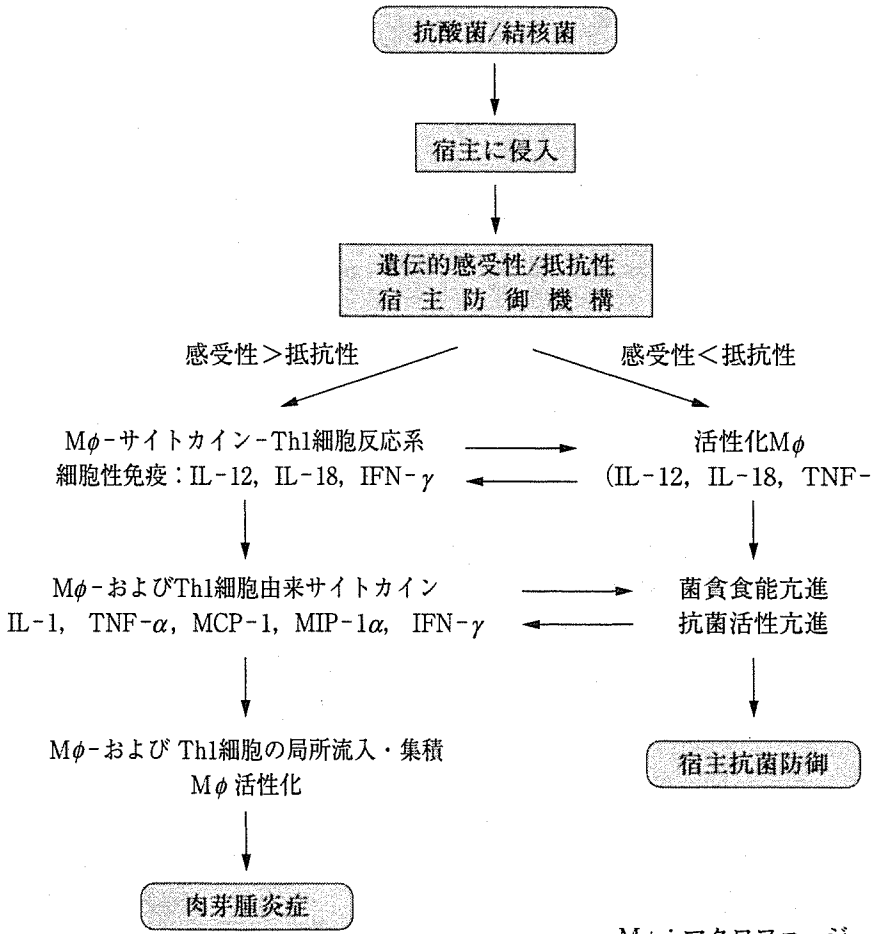
肉芽腫炎症は、発症機序により異物性(T細胞非依存性)および過敏性(T細胞依存性)に大別(図1)されるが、結核菌の病原因子と宿主応答について解析した。結核菌TDMを無胸腺ヌードマウスや未免疫マウスに投与することにより、肉芽腫を惹起できること、TDM免疫マウスではTDM誘導肉芽腫が増強されることや、遅延型過敏反応／細胞性免疫の指標である足蹠腫脹反応が誘導されることから、TDMは異物性ならびに過敏性の両機序を介して肉芽腫を形成する[9)11)]。事実、TDM誘導肉芽腫病変は多量の細胞性免疫起動性サイトカイン(IL-12やIFN-γ)を含み、活動性に伴い消長する。したがって、結核肉芽腫の発現に異物性および過敏性の両機序が複合関与しており、結核肉芽腫は混合性肉芽腫である。

肉芽腫形成には病変局所への血中単球の流入や活性化が必須であり、このため、局所のCC-ケモカイン産生や血管新生が重要な役割を演じる。TDMは血管内皮細胞増殖因子(vascular endothelial growth factor：VEGF)を誘導し、局所の血管新生に寄与している[29)]。

加えて、TDMは宿主免疫担当細胞(胸腺や脾臓)にアポトーシスを誘導し、その結果、細胞内寄生病原体の増殖や生存を困難にし、さらに胸腺内自己反応性T細胞を除去、Th1/Th2細胞の分化を制御することによって自己免疫疾患の発症を防止している可能性がある[30)]。しかし、SLはP-L fusion阻止(宿主細胞内生存)を発揮したが、炎症・免疫惹起やアポトーシス誘導活性をまったく示さず、TDMと対照的である[11)29)30)]。

アシル化trehalose脂質化合物であるTDMは結核菌細胞壁表層に存在し、①結核菌の宿主細胞内生存、②炎症・免疫惹起物質(肉芽腫炎症、遅延型過敏反応や血管新生など)や③アポトーシス誘導活性を発揮する多機能分子(**図4**)であり、結核菌—宿主関係、すなわち結核の病態形成に重要な役割を演じている。

図3　抗酸菌／結核菌感染における宿主細胞および機能分子応答機構



Mφ：マクロファージ

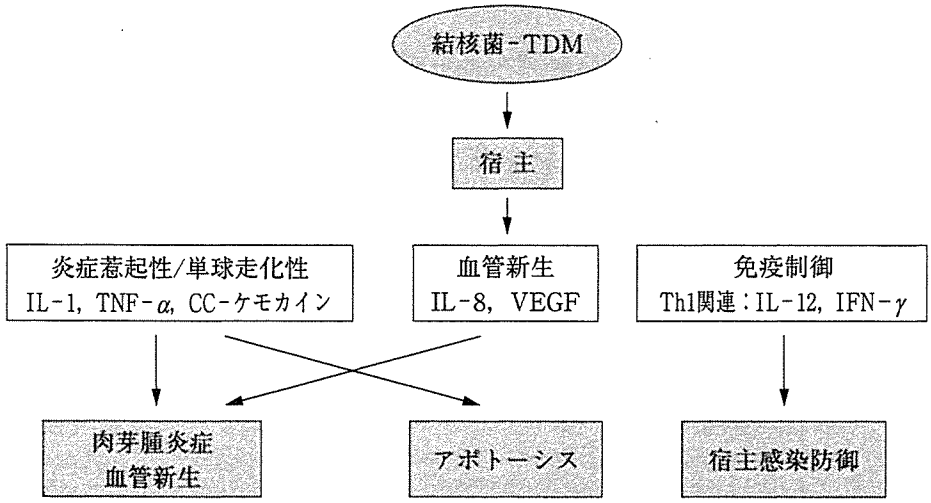
おわりに

結核など抗酸菌感染における宿主防御と肉芽腫炎症機構は菌および宿主側因子が関与する病原体-宿主相互関係（pathogen-host interactions）を介して成立し、抗酸菌と宿主の壮絶な生存戦争を反映している。

肉芽腫炎症は結核菌を局所に封じ込め、感染の拡大を防止し、宿主防御として有益であるが、反面、慢性炎症として自己組織破壊（乾酪壊死や空洞形成）や線維症を伴い、その結果として臓器機能不全を招来し、宿主に不利益を与える側面も有している。

宿主防御や肉芽腫炎症機構の理解は宿主抵抗性を効率的に発現するシステム（ワクチンなど）の開発も促進する[31]。しかし、感染症における宿主防御機構と病態形成は〝諸刃の剣〟、表裏一体の関係に位置している。すなわち、結核制圧において、病原体-宿主関係をよりよく理解することは制圧戦略を構築するために必須である。

図4　結核菌細胞壁TDMと宿主応答の分子機序



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Identification of *Mycobacterium* species by comparative analysis of the *dnaA* gene

Tetsu Mukai, Yuji Miyamoto, Toshio Yamazaki & Masahiko Makino

Department of Microbiology, Leprosy Research Center, National Institute of Infectious Diseases (NIID), Higashimurayama, Tokyo, Japan

Correspondence: Tetsu Mukai, Department of Microbiology, Leprosy Research Center, National Institute of Infectious Diseases (NIID), 4-2-1, Aobacho, Higashimurayama, Tokyo, 189-0002, Japan. Tel.: +81 42 391 8211; fax: +81 42 391 8807; e-mail: tmukai@nih.go.jp

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Mycobacterium spp.; *dnaA* gene; differential diagnosis; LAMP assay.

Abstract

For the establishment of a diagnostic tool for mycobacterial species, a part of the *dnaA* gene was amplified and sequenced from clinically relevant 27 mycobacterial species as well as 49 clinical isolates. Sequence variability in the amplified segment of the *dnaA* gene allowed the differentiation of all species except for *Mycobacterium tuberculosis, Mycobacterium africanum* and *Mycobacterium microti,* which had identical sequences. Partial sequences of *dnaA* from clinical isolates belonging to three frequently isolated species revealed a very high intraspecies similarity, with a range of 96.0–100%. Based on the *dnaA* sequences, a species-specific primer set for *Mycobacterium kansasii* and *Mycobacterium gastri* was successfully designed for a simple loop-mediated isothermal amplification method. These results demonstrate that the variable sequences in the *dnaA* gene were species specific and were sufficient for the development of an accurate and rapid diagnosis of *Mycobacterium* species.

Introduction

Increasing reports of opportunistic infection by nontuberculous mycobacteria (NTM) in immunocompromised patients such as AIDS patients and elderly people are a matter of serious concern to public health (Horsburg, 1991; Montessori *et al.*, 1996; Primm *et al.*, 2004). The routine diagnosis of mycobacteriosis relies primarily on the detection of acid-fast-stained bacilli in the samples by microscopic observation, and the infecting mycobacterial species can be identified with conventional tests including observation of colony morphology and pigmentation, growth rate, and biochemical characteristics (Cernoch *et al.*, 1994; Metchock *et al.*, 1999). Disadvantages of this approach include the time taken to provide clinically relevant information. The clinician must initiate therapy for *Mycobacterium tuberculosis* against NTM infection several weeks before species identification (Montessori *et al.*, 1996), which may increase health care costs, and may reduce the social activity of the patients. Therefore rapid detection and identification of the species level of mycobacteria is required, both to decide whether measures are needed to prevent the spread of the disease and for adequate therapy (American Thoracic Society, 1997).

The mycobacterium species often implicated in NTM infection are *Mycobacterium avium–Mycobacterium intracellulare* complex (MAC), *Mycobacterium kansasii, Mycobacterium chelonae, Mycobacterium abscessus,* and *Mycobacterium xenopi* (Wayne & Sramek, 1992; Metchock *et al.*, 1999; Primm *et al.*, 2004). *Mycobacterium gordonae, Mycobacterium gastri,* or most of the rapidly growing species are rarely pathogenic, but are often encountered as contaminant in clinical samples. Therefore, the discrimination of these species from pathogenic ones is an important diagnostic issue (Primm *et al.*, 2004).

Several studies have been conducted to develop rapid methods based on molecular technique for identifying mycobacterial species in recent years. The DNA sequences reported for such usage are those of 16S rRNA gene (Kirschner *et al.*, 1993; De Beenhouwer *et al.*, 1995; Cloud *et al.*, 2002), *recA* (Blackwood *et al.*, 2000), *rpoB* (Kim *et al.*, 1999), *gyrB* (Kasai *et al.*, 2000), *hsp65* (Plikaytis *et al.*, 1992; Brunello *et al.*, 2001), or 16S–23S internal transcribed spacer (ITS) (De Smet *et al.*, 1995; Roth *et al.*, 1998). The 16S rRNA gene and ITS-based methods are currently widely accepted as rapid and accurate for identifying mycobacteria (Plikaytis *et al.*, 1992; De Smet *et al.*, 1995; Park *et al.*, 2000; Turenne *et al.*, 2001). However, some species have the same sequence or a very high similarity (Kim *et al.*, 1999; Kasai *et al.*, 2000). This fact indicates the need to develop more reliable and user-friendly molecule-based diagnostic tools.

Recently, Notomi *et al.* (2000) have reported a novel nucleic acid amplification method, termed loop-mediated

isothermal amplification (LAMP), that amplifies DNA with high specificity, efficacy, and rapidity under isothermal conditions. The LAMP reaction requires a *Bst* DNA polymerase with strand displacement activity and a set of four specially designed primers that recognize six distinct sequences on the target DNA, the specificity of which should be extremely high. The amplification products are stem-loop DNA structures with several inverted repeats of the target. The advantage of the LAMP method is that the reaction is performed under isothermal conditions of between 60 and 65 °C. As a result, it requires only simple and cost-effective reaction equipment. The LAMP method has emerged as a powerful tool to facilitate genetic testing for various infectious diseases (Enosawa *et al.*, 2003; Iwamoto *et al.*, 2003; Kuboki *et al.*, 2003; Ihira *et al.*, 2004; Parida *et al.*, 2004; Thai *et al.*, 2004).

The purpose of our work is to identify a species-specific region of *Mycobacterium* sp., and to develop a LAMP assay that can differentiate clinically relevant species.

Materials and methods

Bacterial strains and preparation of genomic DNA

The bacteria used in this study comprised 27 strains and 49 clinical isolates as shown in Table 1. All strains except for *Mycobacterium leprae* were cultured on 1% Ogawa medium (Nissui, Tokyo, Japan) at 37 °C. *Mycobacterium leprae* was prepared from infected nude mouse food pad (Shepard, 1960). Genomic DNA was extracted from mycobacterial strains as follows. Mycobacterial cells were resuspended in 1.8 mL of sterile phosphate-buffered saline (PBS) containing 0.1 mm diameter zirconia/silica beads (BioSpec Products Inc., Bartlesville, OK). The mixture was beaded for 20 s with a Beads Homogenizer Model BC-20 (Central Scientific Commerce, Tokyo, Japan), transferred to a 1.5 mL microcentrifuge tube, and the genomic DNA was purified with proteinase K treatment and phenol/chloroform extraction followed by ethanol precipitation, then suspended in 100 μL distilled water.

Table 1. *Mycobacterium* species and strains used in this study and results of the loop-mediated isothermal amplification assay

Species	Strains	Accession number	Primer set	
			Kan32	Gas583
Mycobacterium abscessus	JATA 63-01 (ATCC 19977)	AB087684	–	–
Mycobacterium africanum	KK 13-02 (ATCC 25420)	AB087685	–	–
Mycobacterium avium	JATA 51-01 (ATCC 25291)	AB087686	–	–
	Clinical isolate 22 strains			
Mycobacterium bovis	JATA 12-01 (ATCC 19210)	AB087687	–	–
Mycobacterium chelonae	JATA 62-01 (ATCC 35752)	AB087688	–	–
Mycobacterium fortuitum	JATA 61-01 (ATCC 6841)	AB087689	–	–
Mycobacterium gastri	KK 44-02 (ATCC 15754)	AB087690	–	+
Mycobacterium gordonae	JATA 33-01 (ATCC 14470)	AB087691	–	–
Mycobacterium intracellulare	JATA 52-01 (ATCC 13950)	AB087692	–	–
	Clinical isolate 17 strains			
Mycobacterium kansasii	KK 21-01 (ATCC 12478)	AB087693	+	–
	Clinical isolate 10 strains		+	–
Mycobacterium leprae	Thai-53	AB087694	–	–
Mycobacterium malmoense	JATA 47-01 (ATCC 29571)	AB087695	–	–
Mycobacterium marinum	JATA 22-01 (ATCC 927)	AB087696	–	–
Mycobacterium microti	KK 14-01 (ATCC 19422)	AB087697	–	–
Mycobacterium nonchromogenicum	JATA 45-01 (ATCC 19530)	AB087698	–	–
Mycobacterium parafortuitum	ATCC 25807	AB087699	–	–
Mycobacterium phlei	ATCC 19249	AB087700	–	–
Mycobacterium scrofulaceum	JATA 31-01 (ATCC 19981)	AB087701	–	–
Mycobacterium simiae	KK 23-08 (ATCC 25275)	AB087702	–	–
Mycobacterium smegmatis	JATA 64-01	AB087703	–	–
Mycobacterium szulgai	JATA 32-01	AB087704	–	–
Mycobacterium terrae	KK 46-01 (ATCC 15755)	AB087705	–	–
Mycobacterium triviale	KK 50-02 (ATCC 23292)	AB087706	–	–
Mycobacterium tuberculosis	JATA 11-01 (H37Rv)	AB087707	–	–
Mycobacterium ulcerans	KK 43-01	AB087708	–	–
Mycobacterium vaccae	KK 66-01	AB087709	–	–
Mycobacterium xenopi	KK 42-01 (ATCC 19250)	AB087710	–	–

All strains were kindly donated by Dr Kashiwabara, NIID.

Clinical isolates were identified by Amplicore *Mycobacterium* kit (Roche Pharma, Basel, Switzerland) or conventional biochemical test (Jamal *et al.*, 2000).

Amplification of the region within *dnaA* gene

Highly polymorphic regions flanked by conserved regions were identified by aligning the *Mycobacterium* spp. *dnaA* sequences, which were available in GenBank at the time this study was initiated. These regions were used to design a pair of degenerate primers, U1F 5′-GTS CAR AAC GAR ATC GAR CG-3′ and U1R 5′-CCB GAY TCR CCC CAG ATG AA-3′. A schematic representation of the primer design is shown in Fig. 1a. PCR was performed in a TAKARA Thermal Cycler MP (TAKARA Biomedical, Otsu, Japan) with a reaction mixture consisting of 1 μL of genomic DNA, each deoxynucleoside triphosphate at a concentration of 200 μM, each primer at a concentration of 0.4 μM, 1 × PCR buffer with 1.5 mM $MgCl_2$ (TAKARA Biomedical), and 1.25 U of ExTaq (TAKARA Biomedical), with 10 μL PCRX Enhancer System solution (Gibco BRL, Rockville, MD) in a total volume of 50 μL. The PCR thermocycles were 3 min at 94 °C, followed by 30 cycles of 94 °C for 10 s, 50 °C for 20 s, and 72 °C for 45 s, with a final extension step at 72 °C for 7 min. PCR products were visualized by UV illumination of an ethidium bromide-stained 1.5% agarose gel and cut out to purify with EASYTRAP Ver.2 (TAKARA Biomedical) according to the manufacturer's instruction.

DNA sequencing and sequencing analysis

The ABI Prism BigDye Terminator v3.1 Cycle Sequencing Kit (PE Biosystems, Foster City, CA) was used for the sequencing of the PCR products. The same primers for amplification were used for sequencing. The sequencing reaction was performed in accordance with the instruction of the manufacturer. Sequencing products were purified with a Centrisep column (Princeton Separations, Adelphia, NJ).

The sequencing output was analyzed by using the DNA Sequence Analyzer computer software (PE Biosystems). The partial *dnaA* sequences were aligned using the Clustal W algorithm (Thompson *et al.*, 1994) of the software DNASpace ver. 3.5 (Hitachi Software Engineering, Yokohama, Japan), and the alignment was manually corrected. A phylogenetic tree was generated by DNASpace ver. 3.5 (Hitachi Software Engineering) with a total of 1000 bootstraps. Pairwise similarity of the partial *dnaA* sequences was determined by using DNASIS package (Hitachi Software Engineering).

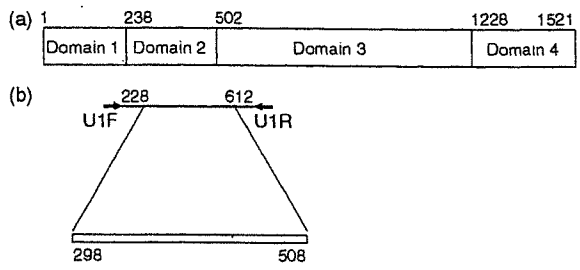


Fig. 1. Schematic representation of the DnaA protein and primer design for the amplification of the partial mycobacterial *dnaA* gene. Number indicates the nucleotide position of *Mycobacterium tuberculosis*, GenBank accession number AL021427. (a) The DnaA protein from *M. tuberculosis* contains four domains. Domain 1 is involved in interaction with DnaB. Domain 2 constitutes a flexible loop. DNA unwinding required Domain 3. Domain 4 is sufficient for specific binding to DNA. Primers U1F and U1R were used to generate about 400 bp fragment from *dnaA* of 27 mycobacterial spp. (b) Analysis and comparison region used in this study are indicated by a bar (298–508 bp).

Species-specific LAMP assay for *Mycobacterium kansasii* and *Mycobacterium gastri*

A set of four primers comprising two inner primers and two outer primers that recognized six distinct regions on the target sequence were designed with PrimerExplorer Ver.3 (Fujitu, Tokyo, Japan). The detailed sequences of the primers are shown in Fig. 3. The two inner primers are called the forward inner primer (FIP) and the backward inner primer (BIP), and each contains two distinct sequences corresponding to the sense and antisense sequences of the target DNA, one for priming in the first stage and the other for self-priming in late stages. FIP contains the sequence complementary F1 (F1c) and F2. BIP contains the complementary B1 (B1c) and B2. The two outer primers consist of F3 and B3.

The LAMP reaction was carried out in 25 μL of reaction mixture by using the Loopamp DNA amplification kit (Eiken Chemical Co. Ltd., Tochigi, Japan) containing 2.4 μM (each) FIP and BIP, 0.2 μM (each) of the outer primers, F3 and B3, 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 8 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 0.1% Tween 20, 0.8 M betaine, 1.4 mM (each) of dNTP, 8 U of *Bst* DNA polymerase (New England BioLabs, Beverly, MA), and the template DNA. Amplification was undertaken in 0.5 μL microtubes in a heatblock under isothermal conditions of 63 °C for 60 min, followed by 80 °C for 2 min to terminate the reaction. Positive and negative controls were included in each run, and precautions to prevent cross-contamination were observed. Two microliter aliquots of LAMP products were subjected to electrophoresis on a 4% agarose gel in Tris-borate-EDTA buffer followed by staining with ethidium bromide and were visualized on a UV transilluminator at 302 nm. The specificity of the LAMP-amplified products were further validated by restriction enzyme digestion with *Nae*I and *Hae*II for *M. kansasii* and *M. gastri*, respectively. The diluted genomic DNA was used for determining the sensitivity of the species-specific LAMP assay.

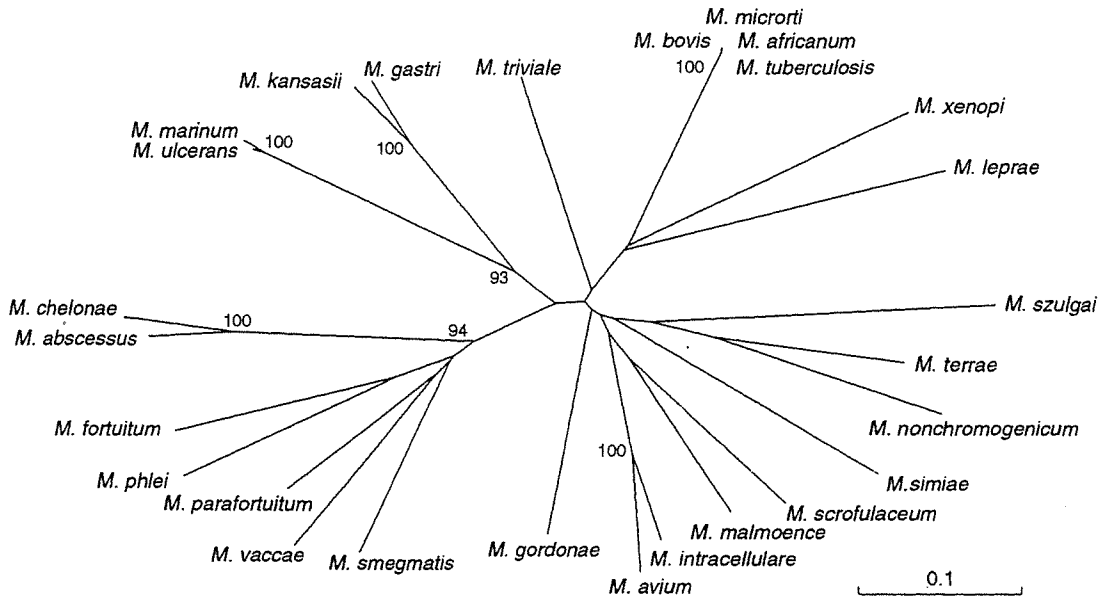


Fig. 2. Phylogenetic relationship of 27 *Mycobacterium* species. Unrooted tree based on the *dnaA* sequences. The tree was generated from DNASpace (Hitachi Software Engineering) with the Clustal W algorithm. The numbers on the dendrogram indicate the percentages of occurrence in 1000 bootstrapped trees; only values of > 90% are shown.

Results

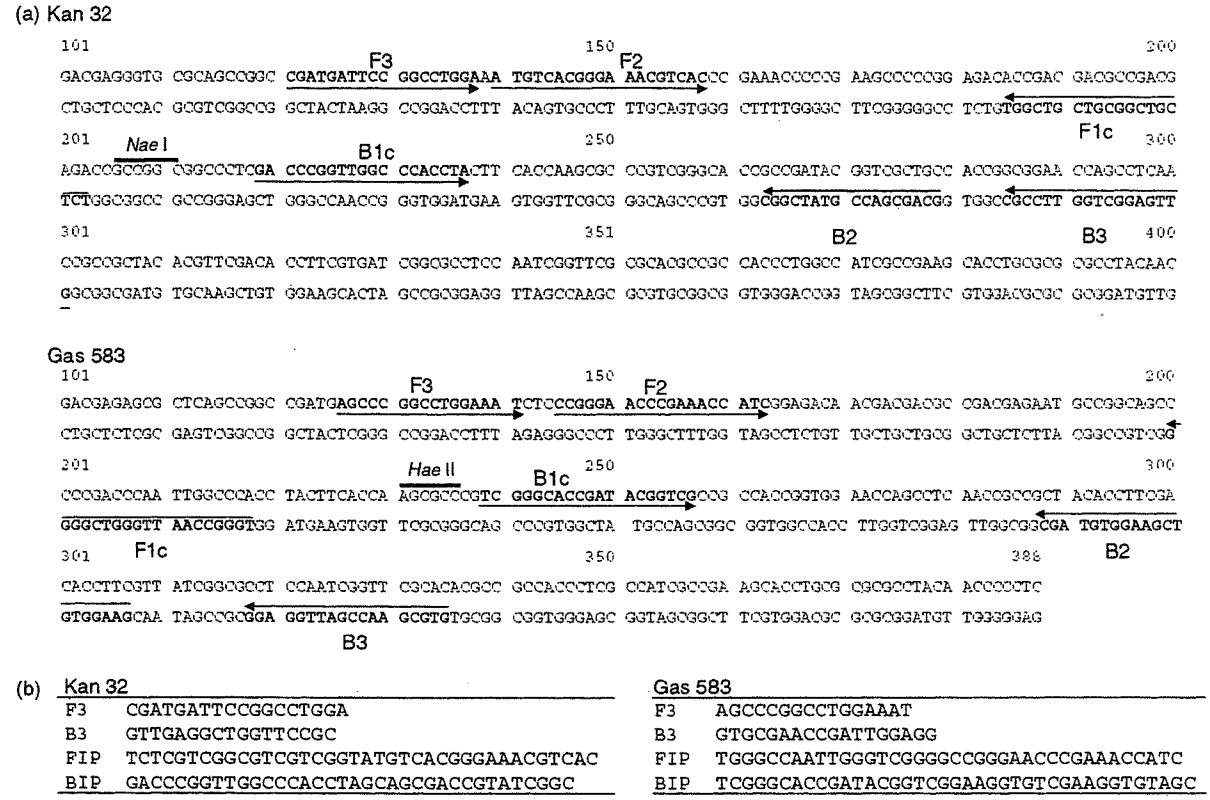
Comparison of partial *dnaA* sequence to identify the *Mycobacterium* species

For the species identification of mycobacterial species, we analyzed some possible variable regions of mycobacterial sequences deposited in the GenBank, and found the 5′ part of the *dnaA* gene as a candidate target for PCR amplification. The PCR products with U1F and U1R, from 27 mycobacterial species, showed the ragged pattern around 400 bp in size (data not shown). Therefore, we determined nucleotide sequences, corresponding to position 228–612 bp of *Mycobacterium tuberculosis*, of all 27 species (Fig. 1a). The alignment of the sequence shows that the region (298–508 bp) in the amplified products had the highest species-specific variability (Fig. 1b). The size of the variable fragment in *dnaA* ranged from 154 bp in *M. triviale* to 232 bp in *M. kansasii*. The variable region exhibits a reasonable number of nucleotide substitution and insertion or deletion sites, which is important for the development of a differential diagnostic tool. The lowest interspecies similarity was 28.2% in *M. leprae* versus *M. vaccae*. The similarity between *M. avium* and *M. intracellulare* was 78.3% and that between *M. marinum* and *M. ulcerans* was 97.7%. Pathogenic *M. kansasii* were easily differentiated from nonpathogenic *M. gastri* (83.6%). The sequences of *M. tuberculosis*, *M. microti*, *M. africanum*, and *M. bovis* were found to be identical, except for one nucleotide substitution that occurred in *M. bovis*. When clinical isolates from clinically relevant mycobacterial strains were analyzed, the following minor variation was found among each species: 97.7–100% (*M. avium*) and 96.0–100% (*M. intracellulare*). We did not find any intraspecies variation in 10 clinical isolates and the standard strain of *M. kansasii*. Because other reports using different systems revealed the existence of more than one sequevar (Yang *et al.*, 1993; Alcaide *et al.*, 1997), we may need to examine a bigger number of clinical isolates.

The unrooted phylogenetic tree showed that the 27 mycobacterial species were resolved by the variable region in the *dnaA* sequence (Fig. 2). All rapidly growing species, *M. abscessus*, *M. chelonae*, *M. fortuitum*, *M. parafortuitum*, *M. phlei*, *M. vaccae*, and *M. smegmatis*, made a cluster that was clearly separated from those of the other species so far examined. On the other hand, *M. kansasii*, *M. gastri*, *M. avium*, and *M. intracellulare* are clinically relevant species; however, the branch of the former two species was obviously segregated from one of the later two species, which was supported by high bootstrap values. The results indicated that the partial *dnaA* sequence could be useful for the differentiation of NTM (Fig. 2).

Identification of mycobacteria by *dnaA* sequence-targeted species-specific LAMP assay

Several sets of primers designed from the *dnaA* sequence were evaluated for their specificity and sensitivity by the LAMP method. One set of primers named Kan-32 for *M. kansasii* and Gas-583 for *M. gastri* was selected (Fig. 3), and



(b)

Kan 32	
F3	CGATGATTCCGGCCTGGA
B3	GTTGAGGCTGGTTCCGC
FIP	TCTCGTCGGCGTCGTCGGTATGTCACGGGAAACGTCAC
BIP	GACCCGGTTGGCCCACCTAGCAGCGACCGTATCGGC

Gas 583	
F3	AGCCCGGCCTGGAAAT
B3	GTGCGAACCGATTGGAGG
FIP	TGGGCCAATTGGGTCGGGGCCGGGAACCCGAAACCATC
BIP	TCGGGCACCGATACGGTCGGAAGGTGTCGAAGGTGTAGC

Fig. 3. Location of oligonucleotide primer sets Kan 32 and Gas 583, used for the loop-mediated isothermal amplification method. For *Mycobacterium kansasii* partial *dnaA* gene (GenBank accession number AB087693) and for *Mycobacterium gastri* partial *dnaA* gene (GenBank accession number AB087690). A right arrow indicates the sense sequence which is used as the primer. A left arrow indicates that a complementary sequence is used as the primer. The unique restriction enzyme recognition sites in the amplified product are shown with a bold bar. (b) List of each primer sequence.

by using these primer sets, a successful LAMP product appeared as a ladder of multiple bands (Fig. 3a).

The species specificity and intraspecies stability of each primer set were examined with purified DNA from 27 mycobacterial species and 10 clinical isolates of *M. kansasii*. We subjected each sample to amplification using Kan-32 or Gas-583 primer set. The results obtained by electrophoretic examination are summarized in Table 1. Although 200 pg of nontargeted species DNA were not amplified, significant amplification of targeted respective isolates was observed after a 60 min incubation at 63 °C. To confirm that the amplification products had corresponding DNA structures, the amplified products were digested with restriction enzymes and the size of the fragments was analyzed by electrophoresis. *Nae*I cuts between F1 and B1c for the *M. kansasii* amplicon; *Hae*II was used for the *M. gastri* amplicons. The sizes of the fragments generated after digestion were in good agreement with sizes predicted theoretically from the expected DNA structure: 100 and 93 bp by *Nae*I digestion, and 123 and 98 bp by *Hae*II digestion (Fig. 4a). Thus, we concluded that each primer set was species specific.

We next assessed the sensitivity of the assay. Serially diluted *M. kansasii* or *M. gastri* genomic DNA was used. The results of a typical experiment are shown in Fig. 4b. Amplified DNA was readily visible when 500 copies of genomic DNA were present in a 60 min incubation assay. The detection limit did not change with a longer incubation period (data not shown).

Discussion and conclusions

For the identification of species, a target gene must be conserved among strains and species. As the DnaA protein is generally conserved among microbial organisms (Mizrahi *et al.*, 2000), this coding region could be used for the target analysis. Four functional domains of the DnaA protein have been defined (Messer *et al.*, 1998). Domain 1 is involved in oligomerization and interaction with DnaB, Domain 2 constitutes a flexible loop, Domain 3 has ATPase function, and Domain 4 is sufficient for specific binding to DNA. The variable region that we identified in the *dnaA* sequence was equivalent to the Domain 2 coding nucleotide sequence

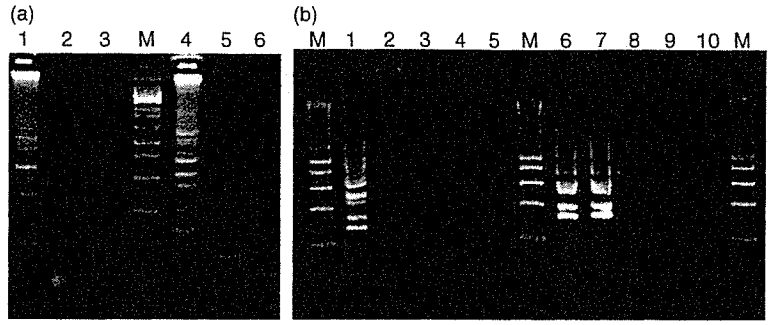


Fig. 4. (a) Four percent agarose gel electrophoresis and restriction enzyme analysis of loop-mediated isothermal amplification (LAMP) products of partial *dnaA* gene of *Mycobacterium kansasii* and *Mycobacterium gastri*. Lanes: M, 100 bp DNA ladder; lanes 1–3, LAMP carried out with *M. kansasii* primer, Kan 32, in the presence of genomic DNA from *M. kansasii* (lanes 1 and 2) and *M. gastri* (lane 3); lane 2, LAMP product from lane 1 after digestion with *Nae* I; lanes 4–6, LAMP carried out with *M. gastri* primer, Gas 583, in the presence of genomic DNA from *M. gastri* (lanes 4 and 5) and *M. kansasii* (lane 6). lane 5, LAMP product from lane 4 after digestion with *Hae* II. (b) Serial dilution of purified *M. kansasii* or *M. gastri* genomic DNA was amplified to determine the sensitivities by LAMP. Lanes: M, 100 bp DNA ladder; lanes 1–5 LAMP carried out with Kan 32 primer set in the presence of genomic DNA of *M. kansasii*, lane 1, 1000 copies; lane 2, 500 copies; lane 3, 100 copies; lane 4, 10 copy; lane 5, distilled water. lanes 6–10 LAMP carried out with gas 583 primer set in the presence of genomic DNA of *M. gastri*, lane 6, 1000 copies; lane 7, 300 copies; lane 8, 100 copies; lanes 9, 10 copy; lane 10, distilled water.

(Fig. 1). This domain is the least conserved region in the *dna A* gene with respect to sequence and length among *M. smegmatis*, *M. tuberculosis*, and *M. leprae* (Fsihi *et al.*, 1996). However, comparative studies of this region using 27 mycobacteria have not been reported and, as far as we know, this is the first report indicating the usefulness of the *dnaA* Domain 2 sequence as a differential diagnostic tool.

An accurate and rapid bacterial identification greatly contributes to this field of medication. Several methods based on molecular biological techniques have been reported. The sequences that have been reported include *hsp65*, 16S rRNA gene, and ITS (Plikaytis *et al.*, 1992; De Smet *et al.*, 1995; Springer *et al.*, 1996; Messer & Weigel, 1997; Roth *et al.*, 1998; Brunello *et al.*, 2001). Each gene has several advantages and disadvantages. An excessive degree of variability is found in the *hsp65* gene (Telenti *et al.*, 1993), which may hinder the development of reliable probes. While 16s rRNA gene sequence is identical in *M. kansasii* and *M. gastri* and shows narrow divergenicity within species (Taylor *et al.*, 1997), ITS sequence can be used to distinguish between *M. kansasii* and *M. gastri* (Roth *et al.*, 1998). While *M. kansasii* is a representative pathogenic mycobacteria, *M. gastri* does not induce an apparent disease. The discrimination between these mycobacteria provides useful information to select the appropriate therapy. The percent similarity of ITS between two species was 93% (Roth *et al.*, 1998), and that of the *dnaA* variable region was found to be 83.6%. These observations may indicate the usefulness of the *dnaA* gene for discrimination of these species, at least in complement with ITS.

The recent trend in genetic testing is to make systems fully automatic with high-throughput analysis. Although this may be an ideal approach, it requires expensive equipment as well as a well-trained person in diagnostic laboratories. The LAMP method could be conducted under isothermal conditions ranging from 60 to 65 °C by a single enzyme. The only equipment needed for LAMP reaction is a regular laboratory water bath or a heat block that furnishes a constant temperature around 63 °C. LAMP does not require a thermal cycling step, and an isothermal reaction for a short time (60 min) is enough to amplify the target DNA to a detectable level. As PCR and other molecular biological techniques are conducted in well-equipped laboratories, these methodologies are often impracticable under a field diagnosis.

In this paper, we demonstrated that the *dnaA* region could be an effective new nucleotide region for the diagnosis of NTM infection and that the LAMP method could be applied for a *dnaA* gene-based differential diagnostic tool.

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European Journal of Immunology

Impaired maturation and function of dendritic cells by mycobacteria through IL-1β

Masahiko Makino[1], Yumi Maeda[1], Tetsu Mukai[1] and Stefan H. E. Kaufmann[2]

[1] Department of Microbiology, Leprosy Research Center, National Institute of Infectious Diseases, Tokyo, Japan
[2] Department of Immunology, Max-Planck-Institute for Infection Biology, Berlin, Germany

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Dendritic cells (DC) are pivotal for initiation and regulation of innate and adaptive immune responses evoked by vaccination and natural infection. After infection, mycobacterial pathogens first encounter monocytes, which produce pro-inflammatory cytokines, including IL-1β, TNF-α and IL-6. The role of these cytokines in DC maturation remains incompletely understood. Here, we show that maturation of DC from monocytes was impaired by pretreatment of monocytes with low doses of IL-1β. Under these conditions, *Mycobacterium leprae*-infected DC failed to stimulate antigen-specific T cell responses. Expression of CD86 and CD83 and production of IL-12 in response to lipopolysaccharide and peptidoglycan were diminished. In contrast, these DC functions were not impaired by pretreatment with TNF-α, IL-6 or IL-10. When monocytes were infected with *M. bovis* Bacillus Calmette-Guérin, and subsequently differentiated to DC, the activity of these DC was suppressed as well. Thus, IL-1β acts at early stages of differentiation of DC and impairs biological functions of DC at later stages. Therefore, production of IL-1β by mycobacteria-infected antigen-presenting cells counteracts effective stimulation of innate and adaptive immune responses.

Key words: BCG · Dendritic cell · IL-1β · IL-12

Introduction

Infections by mycobacterial pathogens still cause major health problems globally. *Mycobacterium tuberculosis* kills more adults than any other infectious agent and one third of the world's population is considered infected with this pathogen [1, 2]. *M. leprae* affects skin and peripheral nerves, causing massive body deformation [3, 4]. These pathogenic mycobacteria persist *in vivo* over long periods without being eradicated by the host immune system [5]. The current vaccine against tuberculosis, BCG has only limited protective effects, and no reliable vaccine has been developed against leprosy [6, 7].

Host defense against mycobacteria in human is primarily conducted by type 1 adaptive immune responses, and DC play a major role as APC [8, 9]. To elicit T cell immunity, activation of APC is critical and strongly influenced by pro-inflammatory cytokines [10–12]. Type 1 $CD4^+$ T cells are stimulated by cognate interactions with APC and IL-12 costimulation [13]. IL-12 is preferentially produced by activated DC and its production is associated with the activation of NF-κB [14, 15]. After appropriate stimulation, blood monocytes can mature to DC and 25% of the circulating inflammatory monocytes are estimated to differentiate to DC and 75% to tissue macrophages [16, 17]. Resting

Correspondence: Dr. Masahiko Makino, Department of Microbiology, Leprosy Research Center, National Institute of Infectious Diseases, 4-2-1 Aobacho, Higashimurayama, Tokyo 189–0002, Japan
Fax: +81-42-391-8212
e-mail: mmaki@nih.go.jp

Abbreviations: MLC: *M. leprae*-derived cytosolic fraction · **MMP:** major membrane protein · **PGN:** peptidoglycan

macrophages serve as habitat for mycobacteria [18]. After infection with mycobacteria, mononuclear phagocytes rapidly produce cytokines including IL-1β as first-line mediators of defense [5, 19].

IL-1β is a 17-kDa prototypic pro-inflammatory cytokine. IL-1β is multifunctional and acts on a wide variety of target cells [20]. It induces numerous genes regulated by IL-1β-inducible transcription factors such as NF-κB [21, 22]. Therefore, IL-1β not only participates in the innate immune response, but also influences T cell activity. However, the effect of IL-1β on monocytes undergoing differentiation into DC remains unknown especially with regard to the adaptive T cell response against mycobacteria.

Here, we show that IL-1β in picomolar quantities markedly influences monocyte maturation to DC with profound consequences for subsequent T cell responses. Our findings that mycobacteria impair DC differentiation and function via IL-1β can explain at least partially the persistence of mycobacteria in host cells.

Results

Effects of IL-1β pretreatment of monocytes on DC-mediated T cell stimulation

Monocytes primed with BCG produced significant concentrations of IL-1β at MOI ≥0.06 (Table 1). Similarly, macrophages produced IL-1β in response to priming with BCG although less efficiently than monocytes. These findings raised the question whether IL-1β influences maturation of DC from monocytes.

To this end, we pretreated monocytes with IL-1β and then allowed them to mature into DC (Table 2). DC derived from untreated monocytes stimulated $CD4^+$ T cells after a pulse with *M. leprae* (MOI 20). In contrast, $CD4^+$ T cell stimulation by DC differentiated from IL-1β pretreated monocytes was markedly impaired. Inhibition depended on the IL-1β concentration used for pretreatment of monocytes. Significant inhibition was achieved at ≥ 100 pg/mL IL-1β. Similarly, IL-1β inhibited T cell stimulation when other Ag, including heat-killed *M. leprae* or major membrane protein (MMP)-II were used to pulse DC. IL-4 or IL-10 were not produced by $CD4^+$ T cells under these conditions (data not shown). IL-1α impaired APC functions of DC in a similar manner as IL-1β, whereas other pro-inflammatory cytokines, including TNF-α and IL-6 did not affect Ag-presenting functions of DC.

Phenotype of DC derived from IL-1β-pretreated monocytes

In an attempt to characterize DC derived from IL-1β-pretreated monocytes, we determined surface markers of immature DC (Fig. 1). Immature DC derived from monocytes, which had been pretreated with IL-1β or not, expressed similar or mildly reduced cell surface levels of HLA-ABC, HLA-DR and CD1a, and were devoid of the macrophage marker CD14. Similarly, the ability of immature DC to engulf mycobacteria as assessed by uptake of GFP-expressing BCG did not differ significantly (GFP-expressing *M. leprae* could not be prepared because of the inability of this obligate intracellular pathogen to grow *in vitro*). Subsequently, we determined surface markers of mature DC generated by LPS stimulation (Fig. 2). Expression of HLA-ABC and HLA-DR was not significantly altered by IL-1β pretreatment of monocytes. In contrast, CD86 and CD83 were down-regulated by IL-1β pretreatment, both with respect to surface expression and percent of positive cell numbers (Fig. 2A). These phenotypic alterations were also observed with *M. leprae*-infected mature DC (data not shown). IL-1β did not influence the surface expression of MMP-II, one of the dominant Ag of *M. leprae* [23], on *M. leprae*-pulsed mature DC (Fig. 2B). In addition, IL-1β or BCG pretreatment of monocytes did not alter the expression of TLR2 and TLR4 (Fig. 2C). Finally, IL-1β pretreatment of monocytes did not cause apoptosis of DC (data not shown). Thus, DC differentiated from IL-1β pretreated monocytes were only partially activated.

Table 1. IL-1β production after stimulation of mononuclear phagocytes with BCG[a)]

Cell type	IL-1β (pg/mL) production after stimulation with BCG at MOI:			
	0	0.0625	0.25	1.0
Monocytes	2.2 ± 0.2	23.7 ± 4.6$^{*b)}$	229.4 ± 19.1$^{**b)}$	861.4 ± 22.3$^{***b)}$
Macrophages	3.3 ± 0.4	4.0 ± 1.1	34.6 ± 2.8**	163.3 ± 20.1$^{****b)}$

a) Plastic adherent monocytes, and M-CSF (5 ng/mL)-treated macrophages (1×10^5/well) were stimulated for 24 h with BCG at the indicated MOI. Assays were done in triplicate, and results are expressed as mean ± SD.

b) $^*p$ <0.05 *vs.* control (MOI 0), $^{**}p$ <0.005 *vs.* control (MOI 0), $^{***}p$ <0.0005 vs. control (MOI 0), $^{****}p$ <0.01 *vs.* control (MOI 0).

Table 2. *M. leprae*-specific IFN-γ production by $CD4^+$ T cells stimulated by *M. leprae*-pulsed DC derived from IL-1β pretreated monocytes[a)]

IL-1β pretreatment of monocytes (pg/mL)	*M. leprae* infection of immature DC (MOI)	IFN-γ (pg/mL) secretion by $CD4^+$ T cells after stimulation with DC at ratio (T: DC):	
		20:1	40:1
0	0	0.7 ± 0.0	0.2 ± 0.0
0	20	206.7 ± 15.1	50.7 ± 4.9
30	0	0.4 ± 0.0	0.3 ± 0.1
30	20	192.8 ± 19.3	24.9 ± 11.2
100	0	0.3 ± 0.1	0.6 ± 0.2
100	20	50.9 ± 6.1*[b)]	13.4 ± 0.3*
300	0	0.7 ± 0.4	0.8 ± 0.7
300	20	30.3 ± 2.6*	9.7 ± 1.9**[b)]
1000	0	0.3 ± 0.1	0.4 ± 0.2
1000	20	9.5 ± 2.1*	3.0 ± 0.5*

a) $CD4^+$ T cells (1×10^5/well) were stimulated for 4 days with autologous DC at the indicated T cell:DC ratio. Immature DC differentiated from untreated or IL-1β-treated monocytes were pulsed with *M. leprae* on day 3, treated with LPS (25 ng/mL) on day 4, and were used as APC on day 5. Representative data of three separate experiments are shown. Assays were done in triplicate, and results are expressed as mean ± SD.

b) *$p < 0.0001$ *vs.* control (IL-1β 0 pg/mL, *M. leprae* (MOI 20)), **$p < 0.0005$ *vs.* control (IL-1β 0 pg/mL, *M. leprae* (MOI 20)).

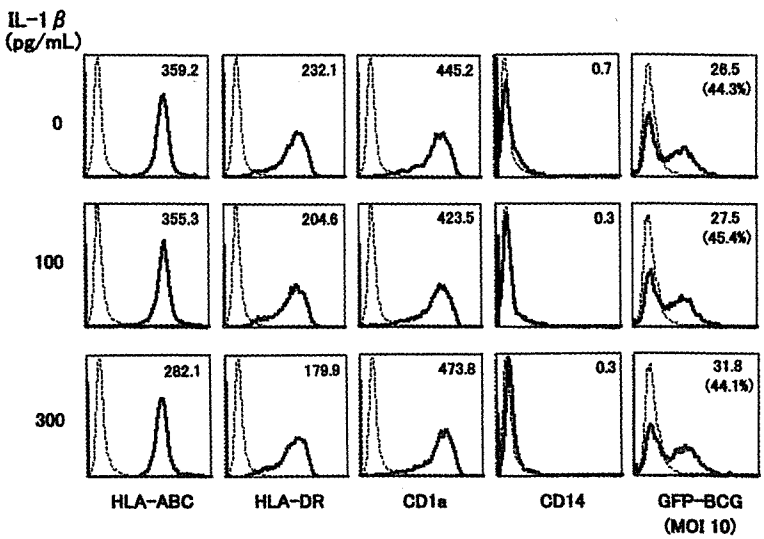

Figure 1. Phenotype and phagocytic activity of immature DC differentiated from IL-1β pretreated monocytes. Plastic adherent monocytes were pretreated with the indicated doses of IL-1β and were subsequently differentiated into DC by 3-day culture with rGM-CSF and rIL-4. For analysis of phagocytic activity of DC, the immature DC (cultured for 3 days) were pulsed with GFP-expressing BCG (MOI 10) and expression of GFP was assessed on day 4 of culture. Dashed lines, isotype-matched control IgG; solid lines, mAb staining. The number represents the difference in mean fluorescence intensity between dotted and solid lines. The number in parentheses indicates percent positive cell number. Representative data of three independent experiments are shown.

Effects of IL-1β pretreatment of monocytes on IL-12 production by DC

In order to determine whether DC differentiated from IL-1β pretreated monocytes can be adequately activated, we determined IL-12p70 production by DC in response to TLR2 and TLR4 signaling. DC obtained on day 4 of culture were found optimal for stimulation by the TLR4 ligand LPS and the TLR2 ligand peptidoglycan (PGN) (data not shown). DC derived from untreated monocytes produced IL-12p70 in response to LPS in a dose-dependent manner, whereas DC differentiated from monocytes pretreated with IL-1β failed to produce significant amounts of IL-12 (Fig. 3A). Similar results

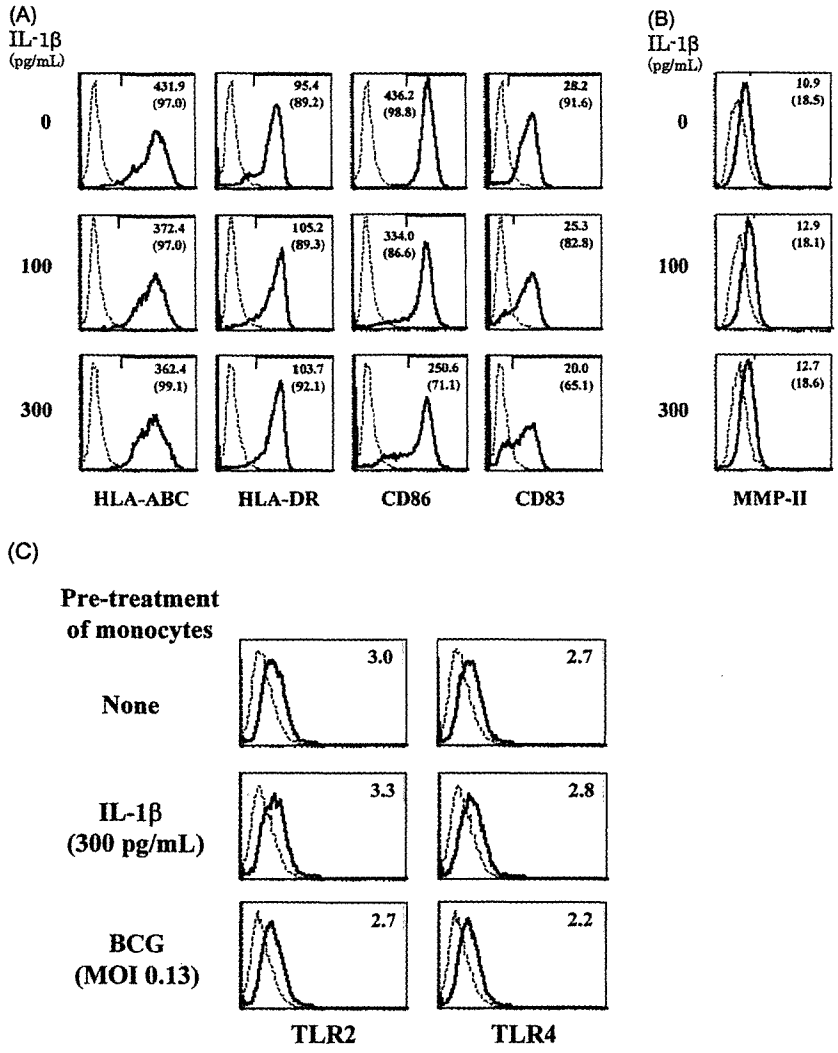

Figure 2. (A) Phenotype of mature DC differentiated from monocytes pretreated with IL-1β. Plastic adherent monocytes were pretreated with the indicated doses of IL-1β and were subsequently differentiated into DC by 5-day culture with rGM-CSF and rIL-4. Cells were treated with LPS (25 ng/mL) on day 4 and analyzed on day 5. Dashed lines, isotype-matched control IgG; solid lines, mAb staining. The number represents the difference in mean fluorescence intensity between dotted and solid lines. The number in parentheses indicates percent positive cell number. Representative data of three independent experiments are shown. (B) MMP-II expression on mature DC obtained as in (A), except that DC were infected with *M. leprae* (MOI 20) on day 3. Dashed lines, isotype-matched control IgM; solid lines, mAb staining. The number represents the difference in mean fluorescence intensity between dotted and solid lines. The number in parentheses indicates percent positive cell number. Representative data of three independent experiments are shown. (C) Expression of TLR on mature DC differentiated from monocytes pretreated with IL-1β or BCG. Plastic adherent monocytes were pretreated with the indicated doses of IL-1β or BCG and were subsequently differentiated into mature DC as in (A). Dashed lines, isotype-matched control IgG; solid lines, mAb staining. The number represents the difference in mean fluorescence intensity between dotted and solid lines. Representative data of three independent experiments are shown.

were obtained for TNF-α production by DC (data not shown). Moreover, untreated DC produced IL-12p70 in response to PGN in a dose-dependent manner, whereas IL-1β pretreatment of monocytes significantly impaired IL-12p70 production (Fig. 3B) although expression of TLR2 on the surface of DC was not altered under these conditions (data not shown). IL-12p70 production in response to MMP-II, which ligates to TLR2, was similarly affected by pretreatment of monocytes with IL-1β (Fig. 3C). Also, both rIL-1β (Fig. 3D) and BCG (Fig. 3E) induced IL-12p70 production only in DC derived from IL-1β untreated-monocytes. The failure to produce IL-12p70 was long lasting as DC differentiated from IL-1β pretreated monocytes in the presence of GM-CSF and IL-4 did not produce IL-12p70 in response to LPS for up to 7 days (data not shown). Next, we examined whether other cytokines produced by monocytes infected with BCG inhibited IL-12 production by DC. Monocytes were pretreated with 100 pg/mL of TNF-α, IL-6 or IL-10, differentiated to DC, which were then stimulated with LPS. None of these cytokines impaired IL-12p70 secretion by DC (Table 3). Thus, IL-1β signaling in monocytes but not IL-6, TNF-α or IL-10 signaling, inhibited IL-12 secretion by DC. Subsequently, IL-1β sensitivity of monocytes undergoing DC maturation was determined. Monocytes were cultured for 3 days in the presence of rGM-CSF and rIL-4 and then treated with IL-1β or other cytokines and 24 h later stimulated with LPS. Cells treated with TNF-α or IL-6 produced similar concentrations of IL-12p70 as compared to untreated DC in response to LPS. In contrast, IL-1β treatment significantly reduced IL-12p70 production (Table 4).

Effects of endogenous IL-1β on IL-12p70 production by DC

We assessed the influence of endogenously produced IL-1β on IL-12p70 production by DC (Table 5). The *M. leprae*-derived cytosolic protein (MLC preparation), which represents an antigenic fraction, did not induce IL-1β production in monocytes at concentrations up to

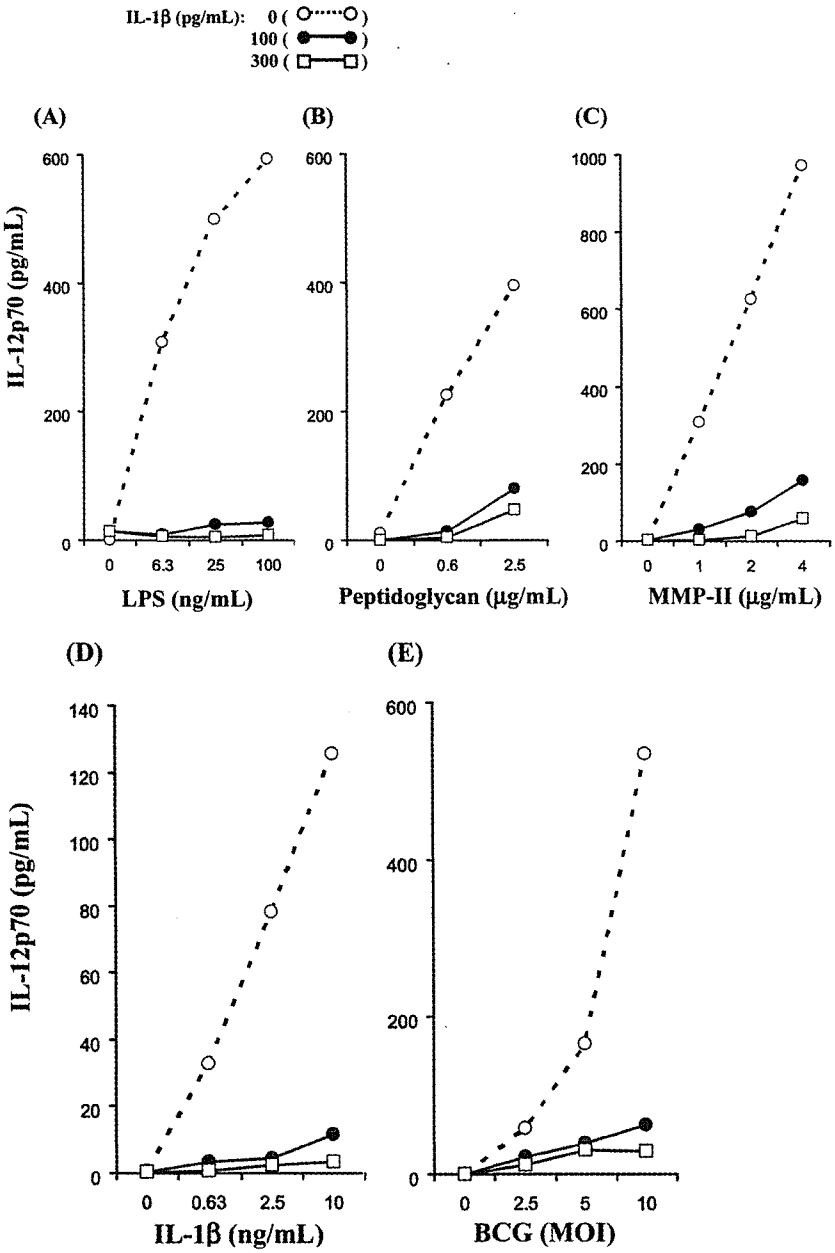

Figure 3. IL-12p70 production by stimulated mature DC. DC were differentiated by 4-day culture with rGM-CSF and rIL-4 from monocytes. Monocytes were treated with the indicated doses of rIL-1β (○----○: IL-1β 0, ●—●: IL-1β 100 and □—□: IL-1β 300 pg/mL), and were stimulated for 24 h with: (A) LPS, (B) PGN, (C) MMP-II, (D) IL-1β and (E) BCG at indicated doses. IL-12p70 concentrations were measured by ELISA. Representative data of three independent experiments are shown.

1 μg/mL, whereas 1 pg/mL LPS stimulated IL-1β secretion in monocytes. Accordingly, DC derived from monocytes pretreated with MLC produced equal concentrations of IL-12p70 as untreated DC did, whereas DC derived from LPS pretreated monocytes failed to secrete significant concentrations of IL-12p70. Monocytes infected with BCG at MOI <0.03 did not produce significant levels of IL-1β and accordingly were able to produce IL-12p70 after differentiation into DC. In contrast, BCG at MOI 0.13 stimulated IL-1β production from monocytes and concomitantly inhibited IL-12p70 secretion by DC. BCG infection did not cause apoptosis under these conditions (data not shown). To directly determine the role of endogenous IL-1β on IL-12 production by DC, antibody neutralization experiments were performed (Table 6). DC derived from BCG-pretreated monocytes were significantly impaired in IL-12p70 production and this impairment was abrogated when neutralizing IL-1β mAb were present during BCG pretreatment. Control mAb showed no neutralizing effects. In addition, impaired IL-12p70 production by LPS pretreated monocytes was abrogated by neutralizing mAb to IL-1β (not shown). Thus, BCG inhibited IL-12 production by DC via IL-1β signaling in monocytes.

Discussion

Host defense against mycobacterial pathogens is mediated by innate and adaptive immune responses.

Table 3. IL-12p70 production by DC differentiated from monocytes pretreated with various cytokines[a)]

Cytokine pretreatment of monocytes (100 pg/mL)	IL-12p70 (pg/mL) production by DC after stimulation with LPS at dose:		
	0	6.3 ng/mL	25 ng/mL
None	4.7 ± 0.8	363.4 ± 6.2*[b)]	546.7 ± 8.7*
IL-1β	3.4 ± 1.0	18.0 ± 2.2*	48.0 ± 5.1*
TNF-α	3.8 ± 0.8	352.8 ± 5.8	531.5 ± 7.3
IL-6	4.0 ± 0.4	375.2 ± 9.8	564.4 ± 11.8
IL-10	1.3 ± 0.1	400.3 ± 11.9	895.8 ± 29.9

a) DC (1×10^5/well) were differentiated from monocytes pretreated with either of the above cytokines on day 0 of culture. On day 4 of culture, DC were stimulated with LPS for 24 h. Representative data of three separate experiments are shown. Assays were done in triplicate, and results are expressed as mean ± SD.

b) *$p < 0.0001$ *vs.* control (LPS 0 ng/mL).

Table 4. Effects of cytokine pretreatment on IL-12p70 production by DC[a)]

Cytokine pretreatment of immature DC (100 pg/mL)	IL-12p70 production (pg/mL) by DC after stimulation with LPS at dose:		
	0	6.3 ng/mL	25 ng/mL
None	0.2 ± 0.0	333.9 ± 11.0*[b)]	539.2 ± 20.3*
IL-1β	0.3 ± 0.0	68.5 ± 9.8*	219.1 ± 10.7*
TNF-α	8.4 ± 1.9	330.7 ± 12.8	534.1 ± 30.0
IL-6	3.6 ± 0.8	331.8 ± 13.4	535.9 ± 29.2

a) Monocytes cultured for 3 days in the presence of rGM-CSF and rIL-4 (1×10^5/well) were pretreated with cytokines and on day 4 cells were further stimulated with LPS for 24 h. Representative data of three separate experiments are shown. Assays were done in triplicate, and results are expressed as mean ± SD.

b) *$p < 0.0005$ *vs.* control (LPS 0 ng/mL).

Table 5. IL-12p70 production by DC derived from Ag-pulsed monocytes[a)]

	Monocyte treatment	IL-1β production (pg/mL) by monocytes[b)]	IL-12p70 production (pg/mL) by DC after stimulation with LPS at dose:		
			0	6.3 ng/mL	25 ng/mL
Exp. 1	None	1.3 ± 0.1	11.8 ± 2.1	250.8 ± 18.7	489.9 ± 21.0
	MLC[c)] (1 μg/mL)	1.6 ± 0.2	13.3 ± 1.8	224.1 ± 17.9	360.9 ± 15.4
	LPS (1 pg/mL)	150.3 ± 3.9	4.0 ± 0.9	1.8 ± 0.3*[d)]	1.5 ± 0.1**
	MLC[c)] (1 μg/mL) + LPS (1 pg/mL)	160.8 ± 4.3	4.4 ± 0.8	0.9 ± 0.2*	0.0 ± 0.0**[d)]
Exp. 2	None	0.0 ± 0.1	0.0 ± 0.0	309.6 ± 20.5	499.3 ± 23.7
	BCG (MOI 0.03)	6.8 ± 0.7	14.6 ± 2.6	264.7 ± 21.8	493.2 ± 21.8
	BCG (MOI 0.13)	148.7 ± 9.6	5.0 ± 1.1	44.6 ± 3.9*	91.8 ± 12.3**

a) DC (1×10^5/well) were derived from monocytes pulsed with Ag or LPS. DC were then stimulated for 24 h with LPS. Representative data of three separate experiments are shown. Assays were done in triplicate, and results are expressed as mean ± SD.

b) The concentration of IL-1β produced by monocytes after priming with indicated stimulator.

c) MLC: *M. leprae*-derived cytosol fraction.

d) *$p < 0.005$ *vs.* None (Monocyte not pretreated), **$p < 0.001$ *vs.* None (Monocyte not pretreated).

Table 6. Effect of IL-1β neutralization on IL-12p70 production by DC[a)]

Monocyte pretreatment		IL-12p70 production (pg/mL) by DC after stimulation with LPS at dose:		
BCG (MOI)	mAb (2 μg/mL)	0	6.3 ng/mL	25 ng/mL
0	None	10.1 ± 0.9	72.4 ± 12.1	301.0 ± 20.0
0.13	None	11.3 ± 0.8	21.5 ± 6.5*[b)]	29.9 ± 9.8**[b)]
0	IL-10	9.1 ± 0.8	80.3 ± 11.9	320.3 ± 21.9
0	IL-1β	12.3 ± 2.1	70.8 ± 9.5	298.0 ± 10.4
0.13	IL-10	13.1 ± 1.9	20.1 ± 7.0*	30.0 ± 10.5**
0.13	IL-1β	10.8 ± 1.3	81.3 ± 9.9	270.3 ± 13.1

a) Monocytes were pretreated with BCG (MOI 0.13) and/or neutralizing mAb to IL-1β or IL-10 (control) (2 μg/mL) and subsequently differentiated into DC by culture with rGM-CSF and rIL-4 for 4 days. DC were stimulated for 24 h with LPS. Representative data of three separate experiments are shown. Assays were done in triplicate, and results are expressed as mean ± SD.

b) *$p < 0.005$ *vs.* control of each column (BCG MOI 0, mAb: none), **$p < 0.0005$ *vs.* control of each column (BCG MOI 0, mAb: none).

The two responses are intertwined and the innate immune response instructs the acquired immune response. Therefore, an effective vaccine should elicit both types of immunity in the most appropriate way [5, 24–27]. DC act as central stimulators of T cells and their maturation and activation is modulated by signals mediated by TLR that sense ligands from microbial pathogens. Subsequently, IL-12 promotes stimulation of protective T cells of Th1 phenotype [12, 28–31]. BCG, *M. tuberculosis* and *M. leprae* preferentially reside in mononuclear phagocytes in which they induce various cytokines including IL-1, TNF-α, IL-6 and IL-10 [32–34]. It is well established that IL-1β is a multifunctional cytokine that plays a key role in the induction of innate and acquired immune responses [20]. In fact, both IL-1β and BCG stimulated massive production of IL-12 from normal DC (Fig. 3). Yet, effects of this cytokine and BCG on maturation of monocytes to DC and subsequent DC activation remain elusive. As shown here, IL-1β pretreatment of monocytes markedly impairs DC maturation and subsequent stimulation of antigen-specific T cells. Moreover, we show that these inhibitory effects are mediated both by exogenous and endogenously produced IL-1β. Phenotype analysis of mature DC revealed that IL-1β impaired surface expression of the costimulatory molecules CD86 and CD83, but had no apparent effect on the phenotype of monocytes and immature DC. Furthermore, secretion of IL-12p70 by mature DC was affected by treatment of monocytes with IL-1β but was not affected by IL-1β-non-inducers, such as LPS-free fraction of *M. leprae* (MLC) (Table 5). TLR signaling induced IL-12p70 production in DC, which, however, was markedly impaired by IL-1β. This was observed when either monocytes or immature DC were treated with IL-1β.

In contrast to IL-1β, treatment of monocytes with TNF-α, IL-6 or IL-10 did not affect IL-12p70 secretion. Moreover, neutralization of endogenously produced IL-1β, but not of IL-10, reversed the inhibitory activity of cytokines produced by BCG-primed monocytes. Thus, IL-1β acts prior to maturation of DC but affects downstream processes in activated DC. Moreover, our data demonstrate that infection of monocytes with BCG stimulates endogenous IL-1β secretion. Thus, interactions between mycobacteria and monocytes affect the development of acquired immunity by impairing secretion of IL-12p70 by BCG-infected monocyte-derived mature DC.

The precise mechanisms underlying inhibition of antigen-specific T cell stimulation by DC via mycobacteria-induced IL-1β remain to be established. It is possible that IL-1β subverts the NF-κB signaling pathway at early stages of DC maturation with downstream consequences on the function of mature DC. Precedences exist, showing that pathogens have developed strategies to circumvent or subvert the NF-κB signaling pathway to promote successful invasion of, and persistence in, the host [35–37]. Moreover, some bacterial pathogens block differentiation of monocytes to DC *in vivo* [38]. An alternative possibility would be induction of apoptosis in DC, a strategy that is, for example, employed by measles virus [39]. However, in our experiments, no evidence for apoptosis of immature or mature DC via IL-1β was obtained. Hence, we consider this possibility less likely. IL-10 is a well-known immunosuppressive cytokine and in HIV infection, IL-10 production severely affects DC maturation resulting in reduced IL-12p70 production and anergic T cell responses [40, 41]. In contrast, our experiments did not reveal evidence for a role of IL-10 in impaired IL-12p70 secretion by DC since neither exogenously added IL-10 on day 0 of monocytes culture nor neutralization of endogenously produced IL-10 caused any apparent effects (Table 6). Thus, IL-1β effects on

monocytes is not dependent on IL-10 expression. One possible mechanism underlying impaired DC function is the attenuation of NF-κB activity. Negative regulation of this pathway could proceed through the induction of negative regulators such as IRAK-M (IL-1 receptor-associated kinases) or other factors at the early stage of monocyte differentiation, which could in turn contribute to impaired TLR signaling by inducing a tolerant phenotype, which hinders production of NF-κB dependent cytokines [42]. Although we consider this explanation likely, the exact mechanism by which IL-1β impairs DC function remains to be elucidated.

Our experiments reveal production of inhibitory IL-1β by monocytes not only after LPS stimulation but also after mycobacterial infection. Our findings provide strong evidence for a critical role of IL-1β in impaired immunity during mycobacterial infections. Hence, they add a guideline for the development of immunological intervention strategies against tuberculosis and leprosy. Strategies that inhibit IL-1β induction or neutralize IL-1β effects at the stage of monocyte maturation to DC could improve the ensuing protective T cell response.

Materials and methods

Preparation of cells and bacteria

Peripheral blood was obtained from healthy PPD-positive Japanese individuals under informed consent. In Japan, BCG vaccination is compulsory for children (approx. 0- to 4-year-old). PBMC were isolated using Ficoll-Paque Plus (Pharmacia, Uppsala, Sweden) and cryopreserved in liquid nitrogen until use, as previously described [43]. For preparation of peripheral monocytes, $CD3^+$ T cells were removed from either freshly isolated heparinized blood, or cryopreserved PBMC using immunomagnetic beads coated with anti-CD3 mAb (Dynabeads 450, Dynal, 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 [44]. Macrophages were differentiated by culturing monocytes in the presence of 20% FCS and 5 ng/mL of M-CSF (R and D Systems, Abingdon, UK), as described [18]. Monocyte-derived DC were differentiated as described [43, 45]. Briefly, monocytes were cultured in the presence of 50 ng of rGM-CSF (Pepro Tech, London, UK) and 10 ng of rIL-4 (Pepro Tech)/mL [45]. Additionally, monocytes were treated with rIL-1β (R and D Systems), rIL-1α (Genzyme, Cambridge, MA), TNF-α (Boehringer Mannheim Biochemica, Mannheim, Germany), rIL-6 (Strathmann Biotech GMBH, Hannover, Germany), or rIL-10 (R and D Systems), and subsequently differentiated into immature DC by culturing the monocytes for 3 days in the presence of rGM-CSF, rIL-4 and either of those cytokines. Mature DC were produced by culturing immature DC, which were unpulsed or infected with *M. leprae*, in the presence of LPS (*Escherichia. coli* 0111: B4; Difco Laboratories, Detroit, MI). *M. leprae* (Thai-53 strain) was isolated from the footpads of BALB/c-*nu/nu* mice [46]. The isolated bacteria were counted by Shepard's method [47]. The viability of *M. leprae* was assessed by using fluorescent diacetate/ethidium bromide test [48]. BCG (Pasteur strain) was cultured *in vitro* using Middlebrook 7H9 broth supplemented with 0.05% Tween 80 and albumin-dextrose-catalase. BCG expressing GFP was constructed as follows: GFP sequence was amplified from pEGFP-1 vector (CLONTECH, Palo Alto, CA), and cloned into pMV261 [49]. Transformants were selected on 7H10 plate containing 25 μg/mL kanamycin. The uptake of BCG by DC after culture was determined using FACScalibur (Becton Dickinson Immunocytometry System, San Jose, CA). The MOI was determined based on the assumption that DC were equally susceptible to infection with *M. leprae* [12], and immature DC were infected with *M. leprae* at MOI 20. The MLC was prepared by fractionation of mycobacterial proteins according to previous reports [18, 28]. Briefly, the mycobacterial suspension was mixed with Zirconium beads and homogenized. The suspension was ultra-centrifuged and the resulting supernatant was used as MLC. The amount of LPS in the purified protein was quantitated by the Limulus Amebocyte Lysate assay (Whittaker Bioproducts, Walkersville, MD) and found to be <10 pg/mg protein.

Analysis of cell surface Ag

The expression of cell surface Ag on DC was analyzed using FACScalibur. Dead cells were eliminated from the analysis by staining with propidium iodide (Sigma Chemical, St. Louis, MO) and 1×10^4 live cells were analyzed. For analysis of cell surface Ag, the following mAb were used: FITC-conjugated mAb against HLA-ABC (G46–2.6, PharMingen, San Diego, CA), HLA-DR (L243, PharMingen), CD1a (OKT6, Ortho Diagnotic Systems, Raritan, NJ), CD14 (M5E2, BD Biosciences, San Jose, CA), CD86 (FUN-1, PharMingen), CD83 (HB15a, Immunotech, Marseille, France), TLR2 (TL2.3, Serotech, Oxford, UK), TLR4 (HTA125, Santa Cruz Biotech, Santa Cruz, CA).

The expression of MMP-II, which is one of the dominant antigenic entities of *M. leprae* [23], on *M. leprae*-infected DC was determined using the mAb (IgM, kappa) against MMP-II, followed by FITC-conjugated anti-mouse Ig . Ab (Tago-immunologicals, Camarillo, CA).

APC functions of DC

The ability of *M. leprae*-infected DC to stimulate T cells was assessed using an autologous DC-T cell co-culture as previously described [12, 45]. Freshly thawed PBMC were depleted of MHC class II^+ and $CD8^+$ cells by using magnetic beads coated with mAb to MHC class II and CD8 (Dynabeads 450; Dynal) [45]. The purity of $CD4^+$ T cells was more than 98%. The purified responder cells (1×10^5 per well) were plated in 96-well round-bottom tissue culture plates and DC were added to give the indicated DC:$CD4^+$ T cell ratio. Supernatants of DC-T cell co-cultures were collected on day 4 and cytokines determined.