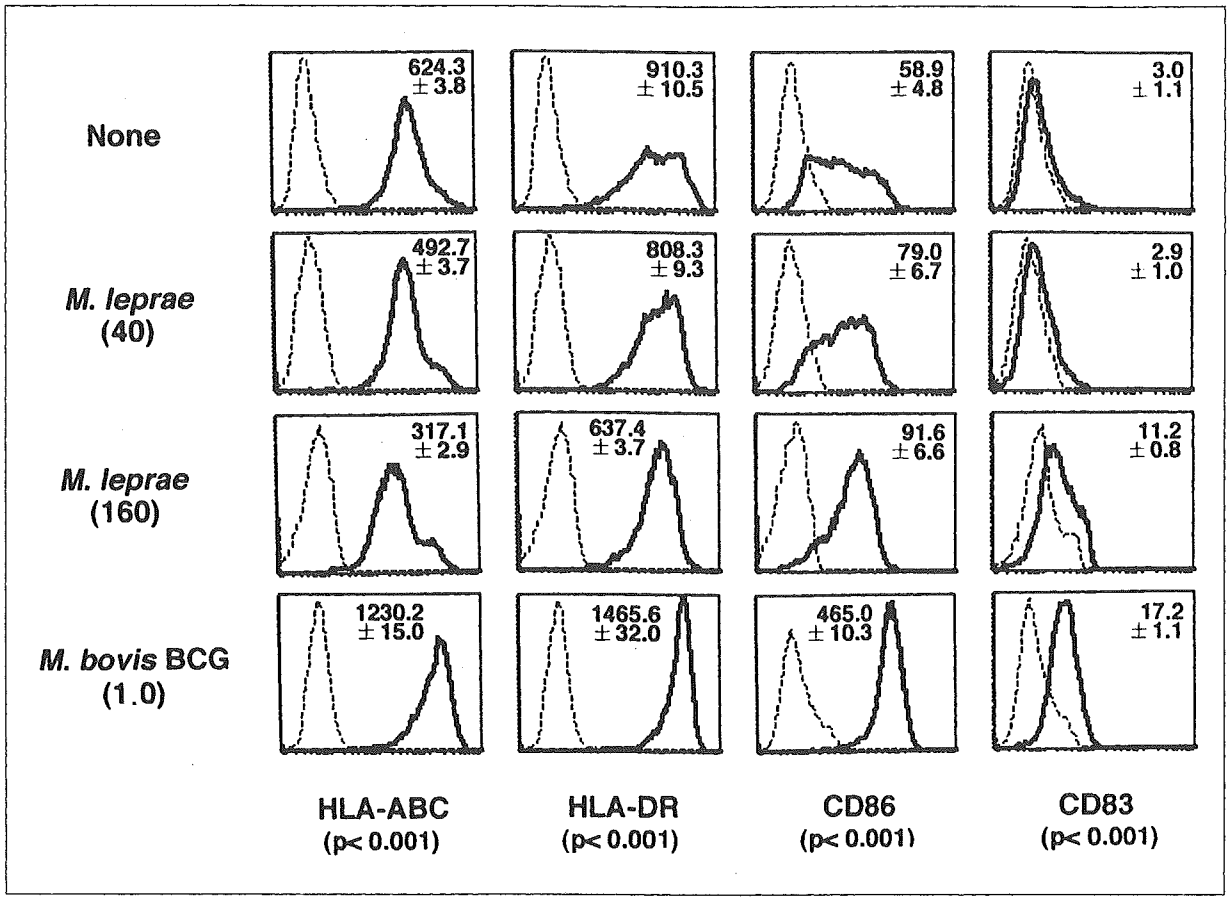

図3　樹状細胞の細胞表面抗原の解析

正常健常者末梢単球より未熟樹状細胞を誘導し，図に示す抗酸菌を感染させた．点線：コントロール抗体，実線：検査抗体．図中の数字はmean fluorescence intensityを示す．

表1　らい菌感染樹状細胞の抗原提示能

Bacteria	Dose (MOI)	Responder ([^{3}H]thymidine uptake ; 10^3cpm)			
		CD4		CD8	
	T/DC :	20	40	10	20
None	NA	7.9±0.8	4.8±0.6	3.0± 0.3	2.7± 0.6
M. leprae					
	10	8.8±0.3	6.3±0.5	3.3± 0.2	4.3± 0.4
	40	25.2±4.1	9.9±1.1	17.2± 0.8	7.4± 0.9
M. bovis BCG	160	29.1±6.7	16.8±2.7	20.1± 1.2	13.5± 1.9
M. avium	0.25	92.4±8.8	65.8±4.9	124.1± 7.9	119.6± 9.1
	1.0	104.6±9.3	66.7±8.1	137.1±10.1	108.2±12.8
	0.25	84.1±6.2	51.1±4.8	61.2± 4.3	33.6± 3.8
	1.0	107.7±7.8	70.2±5.8	67.3± 5.2	51.1± 4.3

レスポンダーT細胞であるCD4陽性およびCD8陽性T細胞(1×10^5/well)を4日間抗酸菌感染樹状細胞で刺激し，[^{3}H]thymidineの取込みを指標にT細胞の増殖応答を検討した．mean±SDを示す．

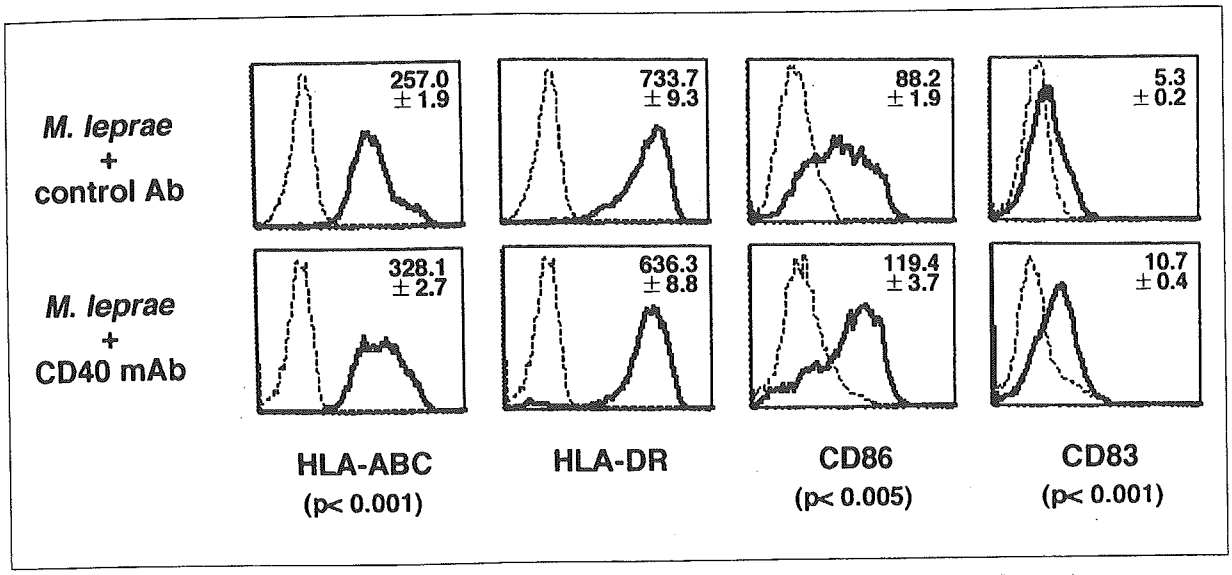

図4　らい菌感染樹状細胞に対する抗CD40モノクローナル抗体の影響
図3と同様にして，らい菌感染樹状細胞を作成し，抗CD40抗体を用いて刺激した．点線：コントロール抗体，実線：検査抗体．図中の数字はmean fluorescence intensityを示す．

表2　らい菌感染樹状細胞表面に発現するPGL-Iの抗原被覆効果

Antigen (MOI)	Responder (T/DC)	Ab(μg/ml) ([³H]thymidine uptake ; 10³cpm)			
		None	DZ2C11 (1.0)	DZ2C11 (0.3)	Control IgG (1.0)
None	CD4(20)	2.2±0.0	3.1±0.2	3.3±0.3	4.2±0.5
	CD8(20)	1.0±0.0	2.0±0.3	0.9±0.4	1.5±0.8
M. leprae (40)	CD4(20)	30.6±1.1	81.1±3.9	97.4±8.1	41.1±5.6
	CD8(20)	3.8±0.9	14.3±1.1	23.1±1.5	7.9±1.2

レスポンダー細胞であるCD4陽性およびCD8陽性T細胞(1×10⁵/well)を抗PGL-I抗体(あるいはコントロールIgG)処理したらい菌樹状細胞あるいは非感染樹状細胞で4日間刺激し，T細胞の増殖を[³H]thymidineの取込みで測定した．

では稀であり，いくつかの原因が考えられる．その点について以下に考察する．

樹状細胞とT細胞の相互作用を抑制する分子の存在

樹状細胞表面のMHC抗原の発現がらい菌感染により充分に増強されなかったことより，らい菌感染樹状細胞は充分活性化していない可能性が示唆された．そこで，モノクローナル抗体を用い，樹状細胞にCD40抗原を介したシグナルを施した．その結果，らい菌感染樹状細胞上のMHC classⅠ, classⅡ, CD86およびCD83抗原の発現は増強し，樹状細胞は活性化した(図4)．しかし，こうした処理によっても，らい菌感染樹状細胞は自己のT細胞の強い活性化を誘導することはできなかった．らい菌にはT細胞のマイトーゲンに対する反応を抑制する物質が存在し，PGL-I抗原がこの抑制性役割をつかさどっていることが報告されている[9]．さらに，らい菌感染によって樹状細胞表面にはPGL-I抗原が発現することから，樹状細胞とT細胞の相互作用が充分に営まれていない可能性が考えられた．そこで，らい菌感染樹状細胞表面に発現したPGL-I抗原を特異抗体を用いて被覆したのちT細胞と接触させたところ，T細胞の増殖応答は増強された(表2)．したがって，らい菌にはたとえ樹状細胞を用いても，また，感染細胞内でプロセッシング作用を受けても，T細胞の増殖を阻止する分子が存在する可能性が明らかになった．しかし，このような抑制性分子を被覆しても他の抗酸菌で観察

された非常に強いT細胞の増殖応答は得られなかったことにより，らい菌そのものの抗原性が脆弱である可能性が残された．近年，らい菌の全ゲノムが解析され，らい菌は結核菌に比べ偽遺伝子が著しく多いことが報告された[10]．らい菌の抗原性が脆弱なこととなんらかの関連があるものと予想される．以下に，らい菌の抗原性について検討する．

らい菌分画の抗原性

らい菌の抗原性について検討を加えるため，らい菌をその構造により細胞壁，細胞膜および細胞質の3つの分画に分画した．これらの分画を樹状細胞にパルスし，その抗原性を検索すると，細胞膜がもっとも強くCD4陽性およびCD8陽性T細胞の増殖応答を誘導した．さらに，細胞膜は，IFN-γの産生を誘導したがIL-4やIL-10の産生は誘導しなかった．したがって，細胞膜はタイプ1T細胞を活性化する能力を有していると考えられた．さらに細胞膜を用いCD8陽性T細胞を10日間刺激し続けると，T細胞内にperforinを産生し，樹状細胞をCD40リガンド存在下で刺激すると大量のIL-12 p70が産生された．しかし，らい菌細胞膜と他の抗酸菌（*M. tuberculosis*, *M. bovis* BCG, *M. avium*）由来の細胞膜の抗原性を比較すると，上記で検査したすべてのパラメーターで，らい菌は他に比し弱いことが明らかになった．そこで，らい菌細胞膜を樹状細胞にパルスしたのち，CD40リガンドを用いて樹状細胞をさらに活性化すると，より強いT細胞応答が得られた．このことから，らい菌細胞膜には，ワクチンあるいは免疫療法に必要な抗原となりうる分子が存在することが明らかになった．

らい菌特異的リポ蛋白の同定

らい菌細胞膜に存在し，らい菌に特異的に発現する分子を同定したならば，ハンセン病に対するワクチン候補分子の開発につながる．

結核菌では19kDのリポ蛋白が同定され，IL-12産生を強く誘導することが報告された[11]．そこで，らい菌のゲノムデーターベースを詳細に検討すると，らい菌特異的リポ蛋白が存在する可能性が判明した．大腸菌を用い，このリポ蛋白（LpK）を発現させ精製したところ，LpKは脂質付加を受けていることが確認され，さらにヒト末梢血単球を刺激して　IL-12産生を誘導することが明らかになった[12]．LpKが樹状細胞を介しT細胞を刺激し，タイプ1CD4陽性T細胞，CD8陽性CTLを産生したならば，ワクチン候補分子となりうる可能性が大きく，今後の解析が期待される．

おわりに

これまでにハンセン病に対するワクチン開発の試みが多方面からなされてきたが，すべて徒労に終わっている．その大きな原因の1つとしてらい菌の脆弱な抗原性があげられる．しかし，樹状細胞を有効利用しCD40リガンドなどの補助因子を活用した上で有効なワクチン候補分子を同定したならば，ハンセン病の新しいワクチンが開発される日も遠くない．

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Assessment of cell mediated immunogenicity of *Mycobacterium leprae*-derived antigens

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Abstract

The antigenicity of *Mycobacterium leprae* (*M. leprae*)-derived cell membrane fraction was examined using human dendritic cells (DCs). Immature DCs internalized and processed the cell membrane components, and expressed *M. leprae*-derived antigens (Ags) on their surface. The expression of MHC class II, CD86, and CD83 Ags on DCs and CD40 ligand (L)-associated IL-12 p70 production from DCs were up-regulated by the membrane Ags. Moreover these stimulated DCs induced significantly higher level of interferon-γ (IFN-γ) production by autologous $CD4^+$ and $CD8^+$ T cells than those pulsed with equivalent doses of live *M. leprae* or its cytosol fraction. Both subsets of T cells from tuberculoid leprosy patients also produced several fold more IFN-γ than those from normal individuals. Furthermore, the intracellular perforin production in $CD8^+$ T cells was up-regulated in an Ag-dose dependent manner. These results suggest that *M. leprae* membrane Ags might be useful as the vaccinating agents against leprosy.

Keywords: Type 1 cellular immunity; Dendritic cells; Antigens; Mycobacteria; $CD4^+$ T cell; $CD8^+$ T cell; IFN-γ

1. Introduction

Mycobacterium leprae is a causative agent of leprosy and cause an irreversible peripheral nerve injury [1,2]. Two to three million individuals are now infected with the bacteria and about half a million new cases are detected each year [3,4]. The recent emergence of *M. leprae* strains resistant to multiple chemotherapeutic agents [5], has necessitated the development of reliable vaccination agents and immunotherapeutic tools.

Host defense against mycobacterial infection such as *M. tuberculosis*, is considered to be mediated primarily by Ag-specific IFN-γ-producing (Th 1 type) $CD4^+$ T cells [6]. Also, mycobacterium-specific $CD8^+$ T cells are recently recognized as the other important component [7,8]. Among $CD8^+$ T cells, cells of Tc1 type, which produce not only IFN-γ but also perforin and granulysin, are considered as direct effector population, because they can induce apoptotic cell death of mycobacteria-infected cells and also directly kill the bacteria [9,10]. The activation of Ag-specific $CD4^+$ and $CD8^+$ T cells is carried out by a cell-to-cell contact with Ag-presenting cells (APCs)[1]. Among the professional APCs, we reported that the *M. leprae*-infected macrophages did not vigorously stimulate autologous T cells [11]. In addition, while the monocyte-derived DCs are known to be the most potent APCs capable of stimulating both naive and memory type T cells and of activating both innate

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[1] *Abbreviations used: M., Mycobacterium*; DCs, dendritic cells; Ag, antigen; mAb, monoclonal antibody; IFN-γ, interferon-γ; L, ligand; APC, Ag-presenting cell; PGL-I, phenolic glycolipid-I; LAM, lipoarabinomannan; CTL, cytotoxic T lymphocytes; PBMC, peripheral blood mononuclear cell; r, recombinant.

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and acquired immunity [12–14], and *M. tuberculosis* and *M. bovis BCG* actually induce the activation and maturation of DCs [15,16], our previous studies indicated that DCs required enormous number of *M. leprae* to stimulate autologous T cells in spite of the fact that DCs can be infected with the bacteria [11]. The exact reason for this less efficient T cell stimulating property of *M. leprae*-infected DCs is still not clear, but, one possible reason may be due to the presence of inhibitory molecules which avoid interaction of DCs with T cells, and of molecules which reduce the expression of MHC class I and class II Ags on DCs. Another reason may be the lack of antigenic molecules on the surface of bacteria, which can evoke drastic T cell responses. However, the latter speculation seems unlikely, since, many of *M. leprae* Ags are supposed to have strong antigenic properties. As a fact, during chemotherapy, some lepromatous patients show strong cellular immune responses against the bacteria, which is recognized as type 1 reverse reaction [17,18].

Presently, not much is known about the antigenic molecules of *M. leprae* in the context of cellular immunity. Recently the whole genome data of *M. leprae* as well as *M. tuberculosis* have been revealed [19,20]. Less than half of the *M. leprae* genome contains pseudogene with intact counterparts in *M. tuberculosis* genome. Therefore, we can presume that *M. leprae* has smaller number of antigenic entities when compared to that of *M. tuberculosis*. In the cell wall fraction of *M. leprae*, phenolic glycolipid-I (PGL-I) is recognized as *M. leprae*-specific Ag [21], but it rather suppresses T cell activation [11,22]. Lipoarabinomannan (LAM), phosphatidylinositol mannosides, and major membrane protein I (MMP-I) are important components of cell membrane fraction of *M. leprae* [23–26]. LAM has activity to evoke CD1b-mediated innate immunity [27], but it skews T cells response to TH 2 type when it binds to C-type lectin on DCs [28].

In addition to the antigenic molecules, the cytokine milieu of both APCs and T cells should be considered, because it can determine the direction of differentiation and activation of T cells. In this respect, IL-12 p70 heterodimer is known to be one of the most important cytokines [29]. However, again, contrary to *M. tuberculosis*, the molecules of *M. leprae* that efficiently induce IL-12 production are not fully uncovered.

The present study was undertaken to search *M. leprae*-derived antigenic molecules, which could be a candidate of vaccinating agent and to assess the antigenicity of such bacterial fraction. We fractionated *M. leprae* into membrane and cytosol components, and examined their antigenicity from the aspect of induction of type 1 $CD4^+$ and $CD8^+$ T cell activation. To this end, we have measured IFN-γ production as a marker of the T cell activation and perforin production as a marker of cytotoxic T lymphocytes (CTL) differentiation.

2. Materials and methods

2.1. Preparation of cells and bacteria

Peripheral blood was obtained under informed consent from healthy, but PPD positive individuals and three tuberculoid leprosy patients. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll–Paque Plus (Pharmacia, Uppsala, Sweden) and cryopreserved in liquid nitrogen until use, as previously described. Monocyte-derived DCs were differentiated from peripheral plastic-adherent cells as described [30,31]. Briefly, $CD3^+$ T cells were removed from either freshly isolated heparinized blood, or cryopreserved PBMCs using immunomagnetic beads coated with anti-CD3 mAb (Dynabeads 450, Dynal, Oslo, Norway). The $CD3^-$ fraction of the PBMCs were plated on collagen-coated plates and cultured for 60 min at 37 °C. The plastic-adherent cells were cultured in 3 ml of RPMI 1640 medium containing 10% FCS and 100 U/ml Penicillin G (Katayama Chemical, Osaka, Japan) for 5 days in the presence of 50 ng of recombinant (r)GM-CSF (Pepro Tech EC, London, England) and 10 ng of rIL-4 (Pepro Tech) per ml. rGM-CSF and rIL-4 were supplied every 2 days as described previously [30,31]. In some cases, Ag-pulsed or unpulsed DCs were further treated with indicated doses of the soluble form of CD40L (kindly supplied by Immunex, Seattle, WA).

Since *M. leprae* cannot be cultivated or grown in in vitro, *M. leprae* (Thai 53) was isolated from the liver of *M. leprae*-infected armadillo. The purified bacteria were counted by Shepard method [32], and were frozen at −80 °C until use. The viability of *M. leprae* was assessed by using fluorescent diacetate/ethidium bromide test [33]. The fractionation of the mycobacterial proteins was carried out according to previous report [26], with modifications. Briefly, the mycobacterial suspension containing the protease inhibitors was mixed with zirconium beads at a ratio of approximately 1:1 (v/v) and homogenized using Beads Homogenizer Model BC-20 (Central Scientific Commerce, Tokyo) at 1500 rpm for 90 s three to four times. The beads were separated and the suspension was centrifuged at 10,000*g* for 30 min to remove the cell wall fractions. The supernatant was then ultra-centrifuged at 100,000*g* for 1 h. The resulting supernatant was taken as the cytosolic fraction and the pellet was suspended in PBS, washed two times and taken as the membrane fraction. This fraction was assessed by Western blotting and found to contain MMP-I, MMP-II, and LAM, which are reliable markers of the membrane fraction [26].

2.2. Analysis of cell surface and intracellular Ags

The expression of cell surface Ags on DCs was analysed using FACScalibur (Becton Dickinson

Immunocytometry System, San Jose, CA). Dead cells were eliminated from the analysis by staining with propidium iodide (Sigma Chemical, St. Louis, MO). For analysis of cell surface Ags, the following mAbs were used: FITC-conjugated mAbs against HLA-ABC (G46-2.6, PharMingen, San Diego, CA), HLA-DR (L243, Becton Dickinson), and CD86 (FUN-1, PharMingen); phycoerythrin (PE)-labeled mAbs against CD83 (HB15a, Immunotech, Marseille, France). Purified murine mAbs to PGL-I (DZ2C11, provided by Dr. H. Minagawa, Leprosy Research Center) and LAM (CS-35, provided by Prof. P.J. Brennan, Colorado State Univ.), which was followed by FITC-labeled goat $F(ab')_2$ anti-mouse IgG (Tago-immunologicals, Camarillo, CA). We also used sera donated by leprosy patients (provided by Dr. H. Minagawa), 1 ml of serum from each of 10 patients was pooled and used to detect *M. leprae*-derived Ags, followed by FITC-conjugated murine anti-human Igs (Tago-immunologicals) for detection by FACScalibur. To determine differentiation of T cells to CTL by stimulation with Ag-pulsed DCs, we performed intracellular staining of perforin. Briefly, $CD4^+$ and $CD8^+$ T cells were surface stained with PE-labeled mAb to CD4 and CD8, respectively, and were fixed in 2% formaldehyde. Subsequently, they were permeabilized using Lysing solution (Becton Dickinson) and Permeabilizing solution (Becton Dickinson), and stained with FITC-conjugated anti-perforin mAb (δG9, PharMingen). The optimal concentrations of mAbs and patient's pooled sera were determined in advance.

2.3. Assessment of APC function of DC pulsed with bacterial Ags

The ability of DCs pulsed with various Ags, to stimulate autologous T cells was assessed using an autologous DC-T cell mixed reaction. DCs pulsed with Ags for 48 h were treated with 50 μg/ml of mitomycin C, washed extensively to remove extracellular Ags, and were used as a stimulator. Freshly thawed PBMCs were depleted of MHC class II^+ cells by using magnetic beads coated with mAb to MHC class II Ag (Dynabeads 450; Dynal) and further treated with beads coated with either CD4 or CD8 mAb to select T cells negatively as previously reported [31]. The purity of $CD4^+$ T cells or $CD8^+$ T cells was more than 98%. The purified responder $CD4^+$ and $CD8^+$ T cells (1×10^5 per well) were plated in 96-well round-bottom tissue culture plates and DCs were added at the indicated DC-T cell ratio. The T cell proliferation during the last 10 h of a 4-day culture in the presence of 4% heat-inactivated human serum (a generous gift from Kagoshima Red Cross Blood Center) was quantified by incubating the cells with 1 μCi/well of [^{3}H]thymidine. The results are expressed as mean differences in cpm obtained from triplicate cultures.

2.4. Assessment of cytokine production

Levels of the following cytokines were measured; IFN-γ and IL-10 produced by $CD4^+$ and $CD8^+$ T cells stimulated with DCs infected with *M. leprae* or DCs pulsed with *M. leprae*-derived fractions, and IL-12 p70 produced by DCs stimulated with the Ags in the presence or absence of various concentrations of soluble form of CD40L (Immunex). Supernatants were collected from T cells stimulated with DCs for 4 days or 24-h Ag-pulsed DCs and the concentrations of cytokines were measured using enzyme linked immunoassay kits (Opt EIA Human IFN-γ SET, Opt EIA Human IL-10 SET, and Opt EIA Human IL-12 (p70) SET) available from BD PharMingen International.

2.5. Statistical analysis

Student's *t* test was applied to demonstrate statistically significant differences.

3. Results

3.1. Influence of M. leprae membrane Ags on DCs

The antigenicity of *M. leprae* was assessed using mycobacterial fractions. When immature DCs were pulsed with *M. leprae* and equivalent dose of its cell membrane or cytosol fractions, the DCs pulsed with cell membrane Ags induced significantly higher proliferation

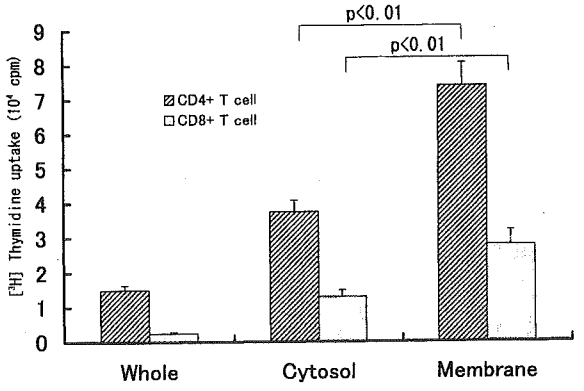

Fig. 1. Proliferative response of autologous $CD4^+$ and $CD8^+$ T cells to DCs pulsed with *M. leprae* or its fractions. The responder $CD4^+$ and $CD8^+$ T cells (1×10^5/well) obtained from healthy individuals were stimulated for 4 days with autologous DCs at a $CD4^+$ T cell:DC ratio of 20 and a $CD8^+$ T cell:DC ratio of 10. Monocyte-derived immature DCs were pulsed with *M. leprae*-derived membrane Ags (16 μg/ml) or with equivalent dose of its cytosol or whole live *M. leprae* (1×10^9 *M. leprae* was taken as 1 mg). One μg per ml of soluble form CD40L was used as a maturation factor of DCs. The T cell activating activity of cytosol and membrane fraction was statistically compared. Representative result of three separate experiments are shown. Assays were done in triplicate, and results are expressed as means ± SD. ▩: $CD4^+$ T cells, □: $CD8^+$ T cells.

of autologous $CD4^+$ and $CD8^+$ T cells than DCs pulsed with the other components (Fig. 1). This point was assessed by using various concentrations of Ags and *M. leprae*, and similar tendencies were observed (not shown). The cell wall fraction, however, was quite toxic to DCs. Therefore, in the following experiments, we chiefly focused on membrane fractions and examined their involvement in the up-regulation of the immune system.

We assessed whether *M. leprae*-derived Ags are expressed on DCs. The membrane Ags pulsed DCs expressed Ags such as LAM, which are known to be present in the cell-membrane fraction, but failed to express cell wall Ag such as PGL-I on their surface (Fig. 2a). In addition to LAM, the DCs pulsed with the Ags positively reacted with the pooled sera obtained from leprosy patients, the expression level of which was significantly reduced in the presence of chloroquine, an inhibitor of intracellular processing of Ags (Fig. 2a). When FITC-conjugated membrane and cytosolic Ags were pulsed to DCs, the cells positively emitted fluorescence and their levels were not reduced after treat-

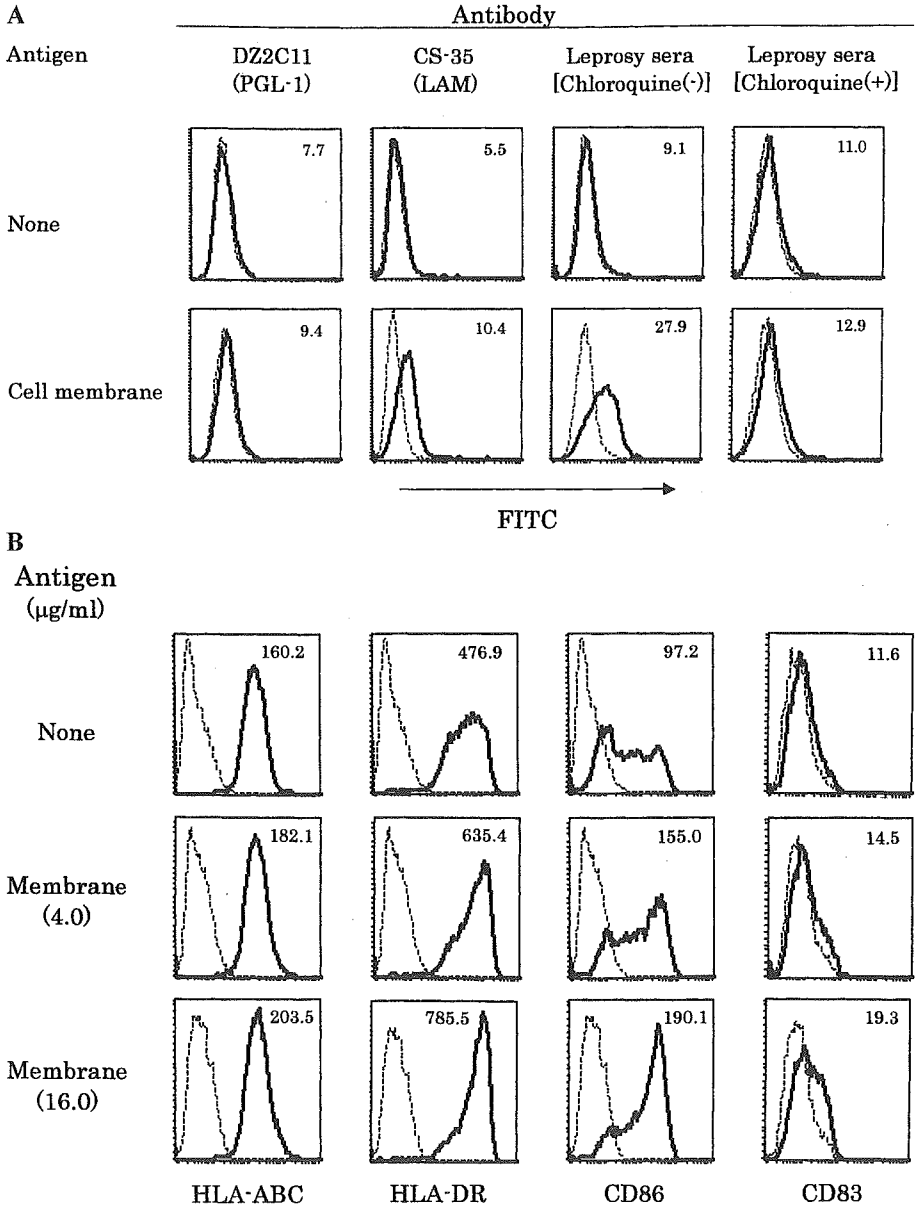

Fig. 2. (A) Surface expression of *M. leprae*-derived Ags on DCs. Monocyte-derived DCs from healthy individuals were pulsed with *M. leprae*-derived membrane Ags (16 μg/ml) in the presence or absence of chloroquine and were stained with indicated mAbs or sera pooled from leprosy patients. - - - - -, control mAb or sera from uninfected donors; ——, indicated mAb or sera from leprosy patients. The mean fluorescence intensity and a representative of three independent experiments are shown. (B) Expression of various molecules on DCs pulsed with *M. leprae*-derived membrane Ags. Monocye-derived DCs were obtained from healthy individuals. - - - - -, control mAb; ——, indicated mAb. The mean fluorescence intensity and a representative of three independent experiments are shown.

ment with trypan blue which quenches the fluorescence due to mere attachment of FITC-conjugated Ags to cell surface (not shown). These results suggest that immature DCs take up *M. leprae*-derived membrane Ags and express their derivatives on the surface of DCs.

We then examined the cell surface APC-associated molecules on DCs. Upon exposure to membrane Ags, immature DCs expressed higher level of MHC class II and CD86 on the surface in the manner dependent on the Ag dose, and CD83 Ag expression was induced when higher dose of membrane Ags was used. Although, a significant up regulation of MHC class I expression was not observed (Fig. 2b). These results suggest that *M. leprae*-derived membrane Ags at least partially induced maturation of DCs.

3.2. Assessment of antigenicity of M. leprae-derived membrane Ags

The APC function of DCs pulsed with membrane Ags was assessed using autologous DC-T cells mixed reactions. The membrane Ags pulsed DCs stimulated both $CD4^+$ and $CD8^+$ T cell subsets, and produced a significant dose of IFN-γ (Table 1). The IFN-γ production was dependent on the dose of Ags pulsed to DCs and on the number of DCs, enrolled in T cell stimulation. Furthermore, when the antigenicity of *M. leprae*-derivatives was compared using IFN-γ as a responder product, membrane Ags induced significantly higher production than cytosol fraction. These phenomena were observed in both $CD4^+$ and $CD8^+$ T cells (Table 1, Exp. 1). On the other hand, since the expression of MHC class I Ag on DC was up-regulated marginally, we determined the effect of maturation factors on DCs [34]. When DCs were pulsed with membrane Ags and further treated with soluble form of CD40L, significant up-regulation of IFN-γ production by both T cell subsets was observed. This up-regulation was significant when 1.0 μg/ml of membrane Ag was used (Table 1, Exp. 2). The CD40L-mediated up-regulation of IFN-γ production by cytosol fraction was also observed (not shown). We then examined the T cell activity towards the Ags in tuberculoid leprosy patients. Marked IFN-γ production was induced in both $CD4^+$ and $CD8^+$ T cells when stimulated with membrane Ags pulsed autologous DCs in all three leprosy patients examined. The representative case is shown in Fig. 3. More than 70% suppression of IFN-γ production was observed, when T cells were stimulated in the presence of mAb to CD86 Ag. We also observed that T cells stimulated with cell membrane-pulsed DCs did not produce significant level of IL-10 (not shown).

We further assessed if membrane Ags pulsed DCs induced $CD8^+$ CTL by measuring the intracellular perforin production (Fig. 4). Consistent with previous study indicating that perforin is constitutively expressed in the granules of cytolytic T cells, both $CD4^+$ and $CD8^+$ T cells stimulated with Ag-unpulsed DCs produced marginal level of intracellular perforin. However, while $CD4^+$ T cells stimulated by the membrane Ag-pulsed DCs did not enhance the perforin production, $CD8^+$ T cells produced significant intracellular perforin by the antigenic stimuli. The $CD8^+$ T cells perforin production was dependent on the dose of Ag, and, as seen in Fig. 4, 25.3% of $CD8^+$ T cells produced perforin

Table 1
IFN-γ production by T cells stimulated with DCs pulsed with *M. leprae*-derived fractions[a]

	Antigen	Dose (μg/ml)	MAF[b]	$CD4^+$ T cells		$CD8^+$ T cells	
				T/DC 20	40	10	20
				(IFN-γ, pg/ml)			
Exp. 1	Membrane	1.0	None	230.6 ± 12.2[c]	55.0 ± 8.5[f]	94.5 ± 5.3[c]	28.2 ± 2.7[e]
	Cytosol	1.0		47.1 ± 6.9[c]	25.1 ± 2.0[f]	78.8 ± 5.2[c]	19.9 ± 1.5[e]
	Membrane	4.0	None	366.4 ± 19.6[d]	60.3 ± 7.3[e]	115.4 ± 9.6[c]	86.6 ± 4.8[c]
	Cytosol	4.0		231.2 ± 11.0[d]	39.8 ± 4.6[e]	91.8 ± 8.4[c]	27.4 ± 2.1[c]
Exp. 2	Membrane	0.25	None	87.6 ± 5.5[d]	21.0 ± 1.6	19.2 ± 1.4	7.2 ± 0.8
		0.25	CD40L	134.3 ± 9.7[d]	26.0 ± 1.9	23.6 ± 1.2	15.7 ± 0.9
	Membrane	1.0	None	141.8 ± 9.9[c]	25.0 ± 2.0[d]	18.4 ± 1.0[e]	18.3 ± 1.1[e]
		1.0	CD40L	452.2 ± 26.3[c]	99.4 ± 7.9[d]	424.2 ± 23.0[c]	32.1 ± 2.8[e]

[a] The responder $CD4^+$ and $CD8^+$ T cells (1×10^5/well) were stimulated for 4 days with autologous DCs at an indicated T cell:DC ratio. Monocyte-derived immature DCs were pulsed with various dose of *M. leprae*-derived components on day 3, treated with maturation factor (CD40L, 1.5 μg/ml) on day 4, and were used as stimulator on day 5. Representative of three separate experiments are shown. Assays were done in triplicate, and results are expressed as means ± SD. The detection limit of IFN-γ is 4.7 pg/ml.

[b] Maturation factor of DC.

[c] $p < 0.001$.

[d] $p < 0.005$.

[e] $p < 0.01$.

[f] $p < 0.05$.

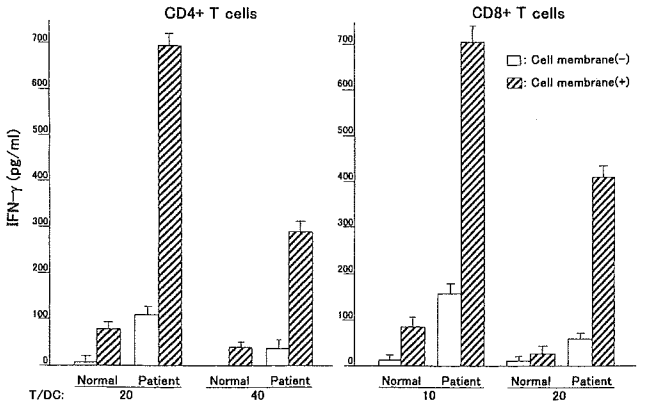

Fig. 3. IFN-γ production of T cells from leprosy patient. The responder $CD4^+$ and $CD8^+$ T cells (1×10^5/well), obtained from healthy donor and tuberculoid leprosy patient, were stimulated for 4 days with autologous DCs pulsed with *M. leprae* membrane Ags (4.0 μg/ml) at an indicated T cells:DC ratio. One μg per ml of soluble CD40L was used as a maturation factor of DCs. Representative result obtained from three different patients is shown. Assays were done in triplicate, and results are expressed as means ± SD.

when higher concentration (16 μg/ml) of membrane Ags was used.

3.3. Production of IL-12 p70 by DC upon stimulation with membrane Ags

The antigenicity of *M. leprae*-derived membrane was further analyzed by measuring the bioactive IL-12 p70 production by DCs (Table 2). Although undetectable level of IL-12 p70 was produced by immature DCs upon membrane or cytosol Ag stimulation, a significant dose of the cytokine was produced when the Ags were pulsed in the presence of soluble CD40L. The IL-12 p70 production was observed with the CD40L treatment alone, of which concentration was dependent on the PBMC donors, but was commonly enhanced by co-pulsing with *M. leprae*-derived Ags. There were significant differences in the cytokine production between membrane and cytosol fractions at two different concentrations of Ags: the former produced significantly higher level than the latter (Exp. 1). When *M. leprae* membrane fraction was further separated into hydrophobic and hydrophilic fraction using Triton X114, both fractions produced IL-12 p70 from DCs (Exp. 2). This result may indicate that *M. leprae*-derived membrane fraction contain various antigenic molecules.

4. Discussion

Leprosy presents a variety of disease spectrum with representative ones: tuberculoid and lepromatous leprosy [35]. The disease manifestation seems to be associated with the impairment of immune responses against *M. leprae*. The differences in the clinical manifestations of leprosy appeared, based on the extent of bacterial spread, which is regulated by the cellular immune responses towards *M. leprae*, and, in this context, IFN-γ-producing type 1 $CD4^+$ T cells and cytotoxic $CD8^+$ T cells occupy a central place [6–8]. Although DC is the

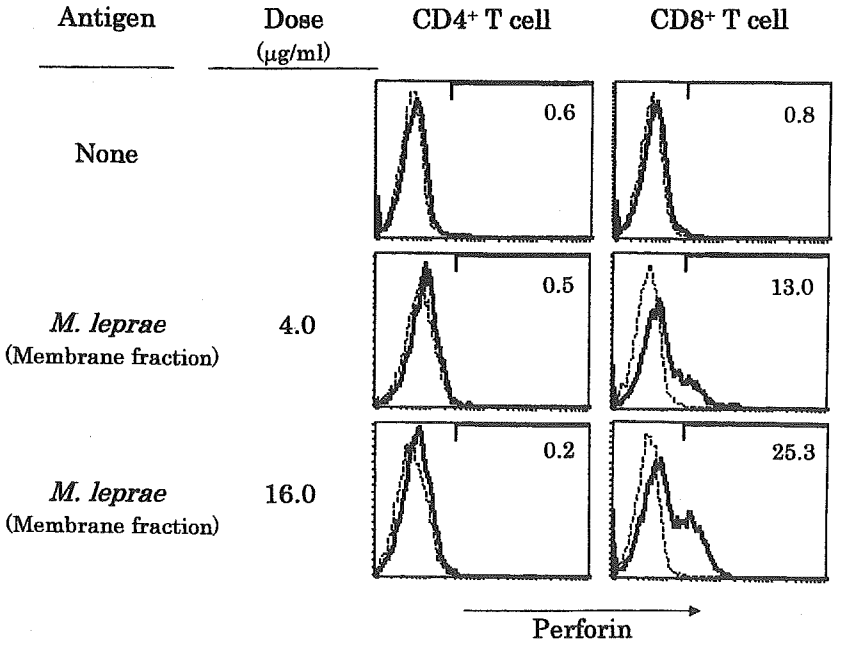

Fig. 4. Intracellular production of perforin. Unseparated PBMCs were stimulated for 10 days with DCs pulsed with *M. leprae* membrane Ags. The $CD4^+$ and $CD8^+$ T cells were gated for analysis and intracellular perforin in both cells was analyzed by using FITC-conjugated mAb to perforin. -----, control mAb; ——, anti-perforin mAb. The number in each figure represents the percent positive cell number among the indicated cell population. A representative of three independent experiments is shown.

Table 2
Production of IL-12 p70 heterodimer by DCs pulsed with *M. leprae*-derived fractions[a]

	M. leprae-derived fraction		Stimulator	IL-12 p70 (pg/ml)
	Fraction	Dose (μg/ml)		
Exp. 1	None		CD40L	68.2 ± 3.1
	Membrane	16	None	ND[b]
	Cytosol	16	None	ND
	Membrane	4	CD40L	217.5 ± 11.9[c]
	Cytosol	4	CD40L	105.4 ± 8.9[c]
	Membrane	16	CD40L	861.5 ± 40.7[c]
	Cytosol	16	CD40L	185.4 ± 9.6[c]
Exp. 2	None		CD40L	49.3 ± 4.6
	Membrane	4	CD40L	151.6 ± 9.3
	Membrane-hydrophobic	4	CD40L	115.2 ± 6.1
	Membrane-hydrophilic	4	CD40L	446.1 ± 25.8
	Membrane	16	CD40L	301.7 ± 20.8
	Membrane-hydrophobic	16	CD40L	251.3 ± 13.2
	Membrane-hydrophilic	16	CD40L	854.9 ± 50.0

[a] Monocyte-derived immature DCs were stimulated for 24 h with various mycobacterial components in the presence or absence of soluble CD40L (1.0 μg/ml). Representative of more than two separate experiments are shown. Assays were done in triplicate, and results are expressed as means ± SD. The detection limit of IL-12 p70 is 7.8 pg/ml.
[b] Not detectable.
[c] $p < 0.001$.

most potent APC to stimulate both $CD4^+$ and $CD8^+$ T cells, we previously reported that DCs required unphysiological number of live or killed bacteria to evoke the activation of these T cells [11]. The results may suggest that subcellular components rather than whole bacteria are useful as vaccinating agents. Therefore, in this study, we examined the subcellular preparations of *M. leprae* for identifying vaccine candidate. However the cell wall fraction consisting of the core of covalently linked mycolic acids, arabinan and galactan attached to peptidoglycan, was quite toxic to DCs probably due to the high lipid and sugar content. Therefore in this paper, we focused on the cytosol and membrane fraction, and concluded that there are more efficient antigens in the membrane fraction, rather than in the cytosol fraction, which should be identified.

M. leprae membrane fraction was taken up and processed by DCs, leading to the up-regulation of MHC class II and CD86 Ags expression. In contrast to whole live *M. leprae*, which reduced MHC class I and class II expression [11], the cellular membrane components of *M. leprae* enhanced the expression level. The exact mechanism leading to the maturation of DCs and enhanced cytokine production is not clear. However, it is likely that toll-like receptors, mannose receptor and DC specific ICAM-3 grabbing non-integrin are involved in the ligation to membrane Ags of *M. leprae* [36,37]. Further study will be required to identify the *M. leprae* membrane ligands which are closely associated with DC maturation. Furthermore, while whole live or killed *M. leprae* expressed molecules which suppress the interaction of DCs and T cells [11], the membrane Ags did stimulate IL-12 p70 production from DCs, IFN-γ production by $CD4^+$ T cells, and both IFN-γ and perforin production by $CD8^+$ T cells. The membrane fraction was superior to cytosol fraction in all these parameters. Although the exact reason for the difference in T cell stimulating activity between two fractions is under investigation, one of the possible reasons for the superiority of membrane fraction may be due to the presence of abundant antigenic molecules. We then investigated whether DC could be differentiated from tuberculoid leprosy patients. From all three patients' monocytes examined, we obtained $CD83^+$, $CD86^+$, MHC class I^+, and class II^+ DCs (not shown), of which phenotype was similar to DCs obtained from healthy individuals (Fig. 2b). When immature DCs were pulsed with membrane Ags, DCs obtained from tuberculoid leprosy patients induced several fold more IFN-γ production from autologous $CD4^+$ and $CD8^+$ T cells than those derived from normal individuals (Fig. 3). This up-regulation of IFN-γ production was observed in all three tuberculoid leprosy patients examined, that suggests that the patients' DCs are able to efficiently present membrane Ags to autologous T cells and that the patient's T cells are primed in vivo with some components of the membrane fraction. There are reports indicating that when T cells from tuberculoid leprosy patients were stimulated with CD3 mAb, they produced significant IFN-γ and also PBMCs from tuberculoid patients produced IFN-γ when stimulated with *M. leprae* antigens [38,39]. Our findings extend the report, and further suggest that Ag-specific IFN-γ production can be induced in these patients by using DCs.

The CD40L molecules are considered, in general, to be an essential component for the induction of type 1 T

cell responses without inducing tolerance of $CD8^+$ T cells [40–42]. In this study, the signaling through CD40L molecules on *M. leprae*-derived cell membrane Ag-pulsed DCs was required for: (a) massive production of IFN-γ by both T cell subsets, (b) production of IL-12 p70 heterodimer by DCs, which is an important cytokine highly associated with bacteriocidal activities [29], and (c) production of perforin by $CD8^+$ T cells. However, LPS did not induce these cytokines to the same level (not shown) and therefore could not substitute CD40L. CD40L may be one of the essential components required for designing the vaccine candidate.

Another important point to be considered is whether $CD8^+$ T cells have cytokilling activity. This point was assessed by measuring the intracellular perforin production. Relation between perforin and $CD8^+$ T cells recognizing *M. leprae* Ags is still unclear. The comparison of *M. leprae* reactive $CD4^+$ T cell line with *M. tuberculosis* recognizing $CD8^+$ T cell line have been reported and the $CD8^+$ T cell line was found to contain perforin [43]. Our in vitro studies indicated that obvious intracellular perforin production was achieved sorely in $CD8^+$ T cells, although not in $CD4^+$ T cells. The results indicate the possibility of $CD8^+$ T cells being involved in anti-microbial effector function. It is interesting to note that, the upregulation of perforin production was observed in about 60% of the PBMC donor examined (not shown). This fact may be related to the previous clinical trial in Malawi which showed that *M. bovis BCG* vaccination was effective in the prevention of leprosy among 50% of the vaccinated population [44]. Also, recent report showed that, both rhesus and cynomolgus monkeys developed progressive tuberculosis, but only in cynomolgus monkey, *M. bovis* BCG vaccination protected toward disease development [45]. Since CTL differentiation of $CD8^+$ T cells requires at least twice stimulations, we may speculate that the perforin production was a consequence of priming of $CD8^+$ T cells with *M. bovis* BCG (childhood *M. bovis* BCG vaccination carried out in most Japanese individuals) and of secondarily stimulation with *M. leprae* membrane Ags. The present studies indicated that *M. leprae* membrane fraction might have efficient cell immunity-inducing molecules, however, further detailed studies from both, the aspect of priming naïve T cells and of reactivation of memory $CD8^+$ T cells should be pursued.

Taken together, the membrane fraction of *M. leprae* contain antigenic molecules which are associated with induction of Th1 $CD4^+$ T cells and Tc1 $CD8^+$ T cells, and could be useful as vaccination agents against leprosy.

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Activation and regulation of Toll-like receptors 2 and 1 in human leprosy

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The expression and activation of Toll-like receptors (TLRs) was investigated in leprosy, a spectral disease in which clinical manifestations correlate with the type of immune response mounted toward *Mycobacterium leprae*. TLR2-TLR1 heterodimers mediated cell activation by killed *M. leprae*, indicating the presence of triacylated lipoproteins. A genome-wide scan of *M. leprae* detected 31 putative lipoproteins. Synthetic lipopeptides representing the 19-kD and 33-kD lipoproteins activated both monocytes and dendritic cells. Activation was enhanced by type-1 cytokines and inhibited by type-2 cytokines. In addition, interferon (IFN)-γ and granulocyte-macrophage colony-stimulating factor (GM-CSF) enhanced TLR1 expression in monocytes and dendritic cells, respectively, whereas IL-4 downregulated TLR2 expression. TLR2 and TLR1 were more strongly expressed in lesions from the localized tuberculoid form (T-lep) as compared with the disseminated lepromatous form (L-lep) of the disease. These data provide evidence that regulated expression and activation of TLRs at the site of disease contribute to the host defense against microbial pathogens.

Toll-like receptors (TLRs) represent one mechanism by which the innate immune system recognizes biochemical patterns displayed by infectious invaders. The specificity of TLRs in mediating responses to defined bacterial ligands has been clearly shown: TLR2 homodimers[1,2] and TLR2-TLR1 heterodimers mediate the response to microbial triacylated lipoproteins[3,4], TLR3 to double-stranded viral RNA[5], TLR4 to lipopolysaccharide (LPS)[6–8], TLR5 to bacterial flagellin[9], TLR2-TLR6 to diacylated lipopeptides[10], TLR7 and TLR8 to imidazoquinolines[11,12] and TLR9 to bacterial CpG DNA sequences[13]. Upon activation, TLRs trigger the release of cytokines and the induction of co-stimulatory molecules that can influence the nature of the adaptive T- or B-cell response[14]. Activation of TLRs also induces antimicrobial pathways that kill intracellular organisms[15].

Leprosy provides an excellent opportunity to investigate mechanisms of innate and adaptive immunity in humans. First, leprosy primarily affects skin, so the lesions are readily accessible to study. Second, the disease presents as a clinical spectrum that correlates with the level of the immune response to the pathogen[16]. Patients with the tuberculoid form are relatively resistant to the pathogen; the infection is localized and the lesions are characterized by expression of the type-1 cytokines characteristic of cell-mediated immunity[17,18]. In contrast, patients with lepromatous leprosy are relatively susceptible to the pathogen; the infection is systemically disseminated and the lesions are characterized by the type-2 cytokines characteristic of humoral responses. To gain insight into the innate immune response in human disease, we investigated the expression and activation of TLRs in human leprosy.

TLR2 and TLR2-TLR1 mediate activation by *M. leprae*

To determine the role of TLRs in mediating cell activation by *M. leprae*, we used human cell lines transiently expressing TLR homodimers (TLR1–10) or heterodimers (TLR2-TLR1, TLR2-TLR6, TLR2-TLR10) with an endothelial leukocyte adhesion molecule–luciferase (ELAM.luc) reporter. These cells were activated

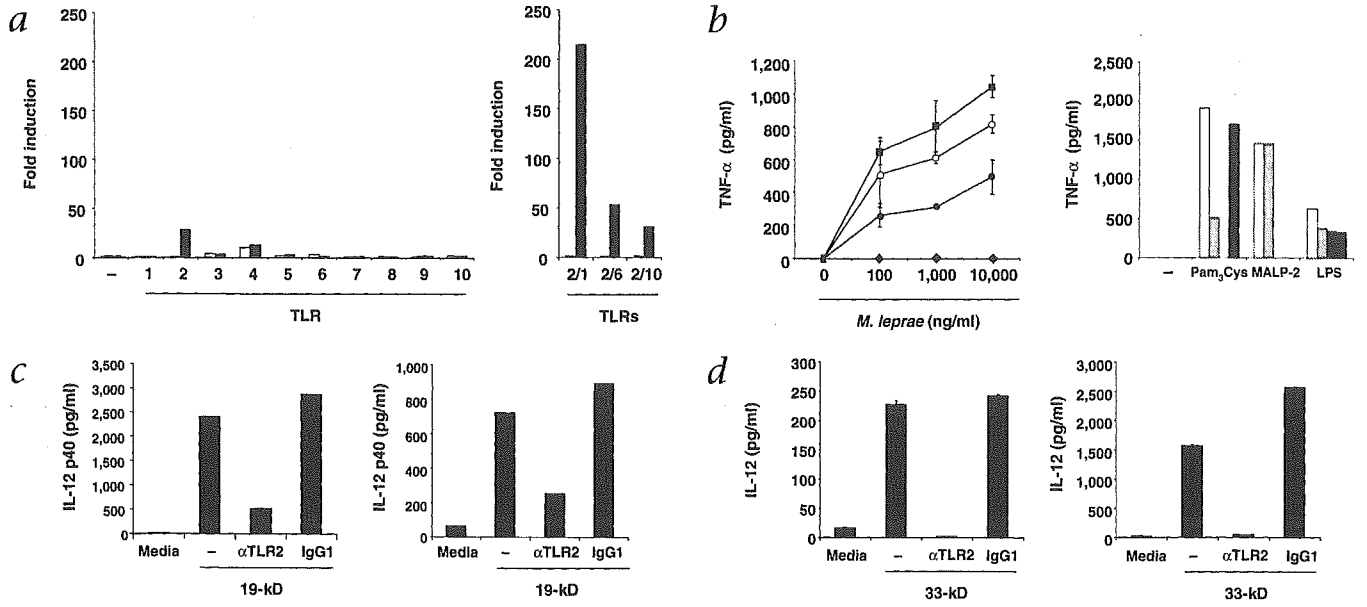

Fig. 1 TLR2 and TLR2-TLR1 mediate the response to *M. leprae* and *M. leprae* 19-kD and 33-kD lipopeptides. ***a***, HEK 293 cells transiently expressing TLR homodimers (TLR1–10) or heterodimers (TLR2/1, 2/6 or 2/10) and an ELAM.luc plasmid were stimulated with killed *M. leprae*. Cells transfected with TLR4 were also transfected with MD2 (a TLR-4–associated protein). ***b***, Left, peritoneal macrophages from wild-type (■), *Tlr1*$^{-/-}$ (●), *Tlr2*$^{-/-}$ (◆) or *Tlr6*$^{-/-}$ (○) mice were activated with either medium or killed *M. leprae*. Right, peritoneal macrophages from wild-type (□), *Tlr1*$^{-/-}$ (▒), *Tlr2*$^{-/-}$ (■), or *Tlr6*$^{-/-}$ (■) were also activated with either media, Pam_3Cys, MALP-2 or LPS to serve as controls. TNF-α production was measured by ELISA. ***c*** and ***d***, Primary human monocytes (left) or monocyte-derived dendritic cells (right) were stimulated with the *M. leprae* 19-kD (*c*) or 33-kD (*d*) lipopeptides in the presence of medium, a TLR2-blocking antibody (αTLR2) or an IgG1 control antibody. IL-12 p40 production was measured by ELISA. The data are representative of at least 2 independent experiments. Error bars represent s.e.m.

with killed *M. leprae* and luciferase activity was measured. Among the homodimers tested, only TLR2 was able to mediate responsiveness toward *M. leprae*, activating the cells by 28-fold above background (Fig. 1*a*). Co-expression of TLR1 greatly enhanced TLR2-mediated activity, resulting in 215-fold activation (Fig. 1*a*). These data indicate that TLR2 homodimers or TLR2-TLR1 heterodimers can mediate cell activation by *M. leprae*. Studies of *Tlr2*$^{-/-}$ and *Tlr1*$^{-/-}$ mice corroborate the transfection data. Peritoneal macrophages from *Tlr2*$^{-/-}$ mice did not respond to *M. leprae*, as measured by TNF-α production, whereas macrophages from *Tlr1*$^{-/-}$ mice had a diminished response (Fig. 1*b*). In accordance with previous studies, macrophages from *Tlr2*$^{-/-}$ mice did not respond to Pam_3Cys or macrophage-activating lipopeptide (MALP)-2. Macrophages from *Tlr1*$^{-/-}$ and *Tlr6*$^{-/-}$ mice had diminished responses to Pam_3Cys and MALP-2, respectively (Fig. 1*b*).

Two groups recently showed that TLR2-TLR1 heterodimers mediate the response to triacylated lipopeptides[3,4]. It was thus logical to investigate *M. leprae* lipoproteins and synthesize corresponding triacylated lipopeptides as a model for *M. leprae* ligands that stimulate TLR2 and TLR1. Unfortunately, *M. leprae* cannot be grown in the laboratory and it is difficult to purify proteins from the small number of organisms that can be harvested from armadillos. We therefore scanned the *M. leprae* genome for proteins with a putative lipoprotein signal motif and identified 31 putative lipoproteins (see Supplementary Table 1 online). The *M. leprae* lipoproteins ML1966 and ML0603 were chosen for further study.

ML1966 (a putative *M. leprae* 19-kD lipoprotein) shares 47% amino acid sequence identity with the 19-kD lipoprotein of *Mycobacterium tuberculosis* (lpqH), a major inducer of monocyte cytokine release through TLR2 (refs. 1, 3). ML1966 seems to have arisen by means of a gene duplication event as its gene is located in a different site than that occupied by *lpqH* (Rv3763) of *M. tuberculosis*. The *M. leprae* chromosomal locus corresponding to Rv3763 contains the pseudogene ML2363, which is strictly orthologous to *lpqH*, and is flanked by pseudogenes equivalent to Rv3762 and Rv3764, the neighboring genes in *M. tuberculosis*. *ML1966* mRNA has been detected in *M. leprae* (D. Williams and T. Gillis, personal communication). ML0603, the *M. leprae* 33-kD lipoprotein, was also studied because expression of this lipoprotein by *M. leprae* has been detected by western blot, and because a recombinant lipoprotein expressed in *Escherichia coli* triggers monocyte cytokine release[19].

Analysis by GC mass spectrometry of mycobacterial lipoproteins provided evidence for the presence of three fatty acid substitutions: two in the glycerol portion (consisting of either palmitic, stearic or tuberculostearic acid) and one in the amide linkage to the cysteine residue (palmitic acid). Although the natural lipoproteins from *M. leprae* may contain a combination of three different fatty acids, tri-palmitylated lipopeptides activate a similar pattern of TLRs when compared with a purified *M. tuberculosis* lipoprotein[3]. Therefore, hexameric peptides with a tripalmityl modification corresponding to the *M. leprae* 19-kD and 33-kD lipoproteins were synthesized and examined for their ability to trigger monocytes in a TLR-dependent manner.

Both *M. leprae* lipopeptides activated primary human monocytes obtained from healthy donors, as measured by the release of IL-12 p40 (Fig. 1*c*). A TLR2-neutralizing antibody blocked activation. Similarly, the 19-kD and 33-kD lipopeptides activated monocyte-derived dendritic cells in a TLR2-dependent manner to release IL-12 p40 (Fig. 1*d*). Together, these data provide evidence that *M. leprae* and two of its constituent ligands activate cells of the innate immune system through TLR2 and TLR2-TLR1.

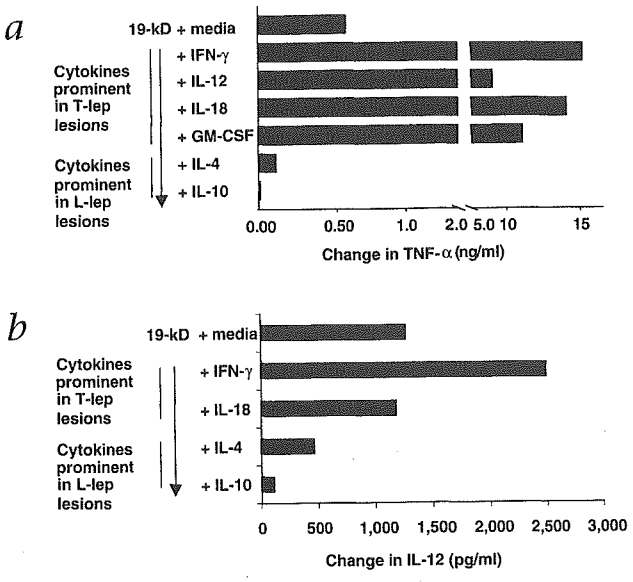

Fig. 2 Cytokine profiles influence TLR2-TLR1 activation of monocyte and monocyte-derived dendritic cells. ***a*** and ***b***, Monocytes (*a*) or monocyte-derived dendritic cells (*b*) were activated with *M. leprae* 19-kD lipopeptide together with either media, IFN-γ, IL-12, IL-18, GM-CSF, IL-4 or IL-10. TNF-α (*a*) or IL-12 p40 (*b*) production was measured by ELISA. The data are shown as TNF-α or IL-12 p40 levels above media background and are representative of 3 independent experiments.

Regulation of TLR2-TLR1 activation by cytokines

For intracellular pathogens, including *M. leprae*, the balance of type-1 and type-2 cytokines influences whether the clinical form of the disease is localized or disseminated, respectively. In leprosy, the type-1 cytokines IFN-γ, IL-12, IL-18, and GM-CSF predominate in localized T-lep lesions, whereas the type-2 cytokines IL-4 and IL-10 characterize disseminated L-lep lesions[17,18]. We investigated whether these cytokines could influence TLR2-TLR1 activation of monocytes and monocyte-derived dendritic cells by stimulating these cells with the *M. leprae* 19-kD lipopeptide in the presence or absence of cytokines, and then measuring cytokine release. The type-1 cytokines IFN-γ, IL-12, IL-18 and GM-CSF enhanced the ability of the *M. leprae* 19-kD lipopeptide to trigger monocyte release of TNF-α (Fig. 2*a*). In contrast, IL-4 and IL-10 substantially inhibited the lipopeptide-induced release of TNF-α. IFN-γ also upregulated lipopeptide-induced release of IL-12 p40 from monocyte-derived dendritic cells (Fig. 2*b*). IL-18 had little effect, whereas IL-4 and IL-10 inhibited IL-12 p40 release. In the absence of the 19-kD lipopeptide, the type-1 and type-2 cytokines did not stimulate monocyte and monocyte-derived dendritic-cell cytokine release (data not shown). These data provide evidence that the local type-1 or type-2 cytokine environment can influence the degree of TLR2-TLR1 activation.

Type-1 and type-2 cytokines influence TLR expression

We next wanted to address whether type-1 and type-2 cytokine

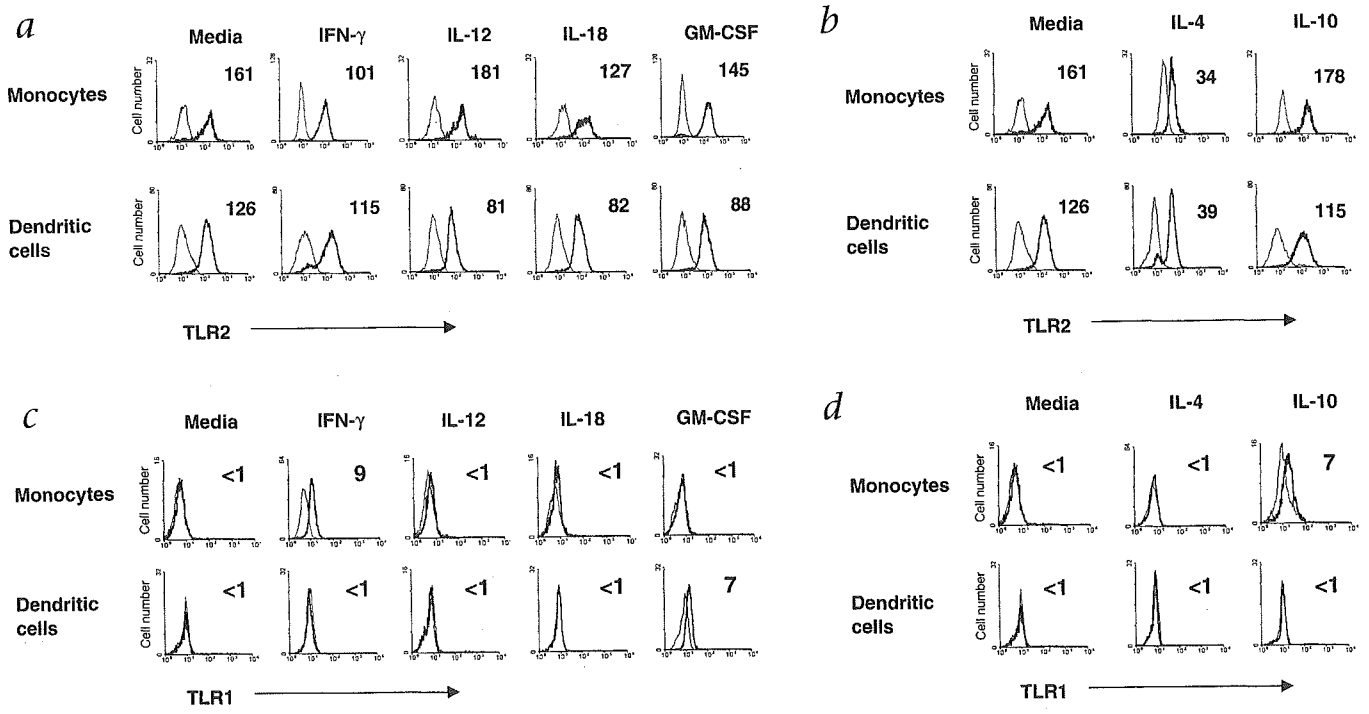

Fig. 3 Effect of cytokines on TLR2 and TLR1 expression on monocytes and monocyte-derived dendritic cells. ***a***, Monocytes and monocyte-derived dendritic cells were treated with media or recombinant type-1 cytokines IFN-γ, IL-12, IL-18 or GM-CSF. Cells were labeled with a TLR2-specific monoclonal antibody or an isotype control and examined by flow cytometry. ***b***, Monocytes and monocyte-derived dendritic cells were treated with media or the recombinant type-2 cytokines IL-4 or IL-10. TLR2 cell-surface expression was examined by flow cytometry. *a* and *b*, the data shown are representative of at least 2 independent experiments; values at upper right of each graph represent changes in mean fluorescence intensity between TLR2 and the IgG1 isotype control. ***c***, Monocytes and monocyte-derived dendritic cells were treated with media, IFN-γ, IL-12, IL-18 or GM-CSF. Cells were labeled with a TLR1-specific monoclonal antibody or an isotype control and examined by flow cytometry. ***d***, Monocytes and monocyte-derived dendritic cells were treated with media, IL-4 or IL-10. TLR1 cell-surface expression was examined by flow cytometry. *c* and *d*, the data shown are representative of at least 2 independent experiments; values at upper right of each graph represent changes in mean fluorescence intensity between TLR1 and the IgG1 isotype control.

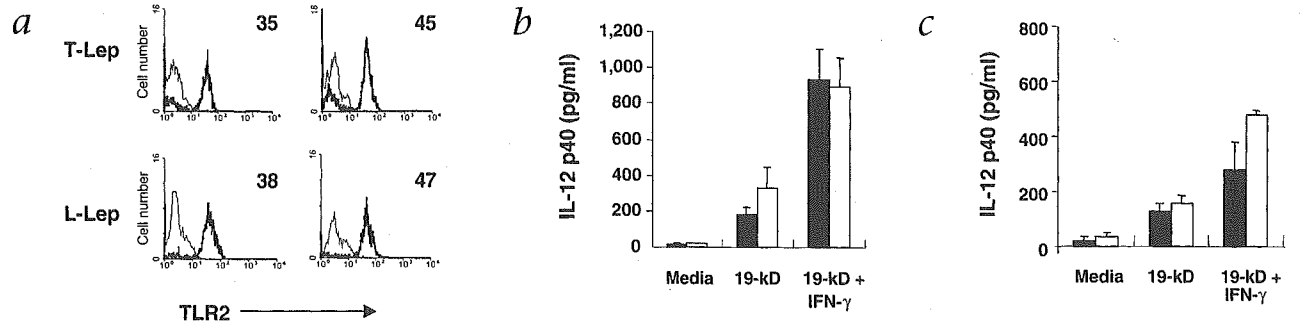

Fig. 4 TLR2 and TLR1 expression on peripheral monocytes, and activation of monocytes and monocyte-derived dendritic cells in leprosy patients. ***a***, Peripheral monocytes were isolated from T-lep and L-lep patients. Expression of TLR2 was measured and represented as change in mean fluorescence between TLR2 and the isotype control. Values in upper right of graph represent change in mean fluorescence intensity. ***b*** and ***c***, Monocytes (*b*) or monocyte-derived dendritic cells (*c*) from T-lep (■; $n = 10$ (*b*) or 4 (*c*)) and L-lep (□; $n = 11$ (*b*) or 6 (*c*)) leprosy patients were stimulated with the 19-kD lipopeptide with or without IFN-γ. IL-12 p40 production was measured by ELISA. Error bars represent s.e.m.

patterns could influence the level of TLR2 and TLR1 expression on monocytes and monocyte-derived dendritic cells. Cells were treated with recombinant cytokines and TLR expression was measured by flow cytometry. The type-1 cytokines IFN-γ, IL-12, IL-18, and GM-CSF did not affect TLR2 expression (Fig. 3*a*). IL-10, which inhibited TLR2-TLR1 activation, did not downregulate TLR2 expression. The type-2 cytokine IL-4, however, substantially decreased TLR2 cell surface expression on both monocytes and monocyte-derived dendritic cells (Fig. 3*b*). As previously shown[20], IL-4 downregulates *TLR2* mRNA as measured by quantitative PCR (data not shown).

In contrast to the downregulation of TLR2 by type-2 cytokines, TLR1 expression was upregulated by type-1 cytokines. Freshly isolated monocytes expressed undetectable to low levels of TLR1, but this expression was increased through the addition of the type-1 cytokine IFN-γ (Fig. 3*c*). The other type-1 cytokines, IL-12, IL-18, and GM-CSF, as well as the type-2 cytokines tested did not influence levels of TLR1 expression. In monocyte-derived dendritic cells, the type-1 cytokine GM-CSF induced expression of TLR1 (Fig. 3*c*). The type 2 cytokine IL-10 seemed to have a modest effect on TLR1 expression in dendritic cells, perhaps from driving the dendritic cell towards a more macrophage-like cell (Fig. 3*d*). Taken together, our data indicate that type-1 and type-2 cytokine patterns differentially regulate TLR1 and TLR2 expression, respectively.

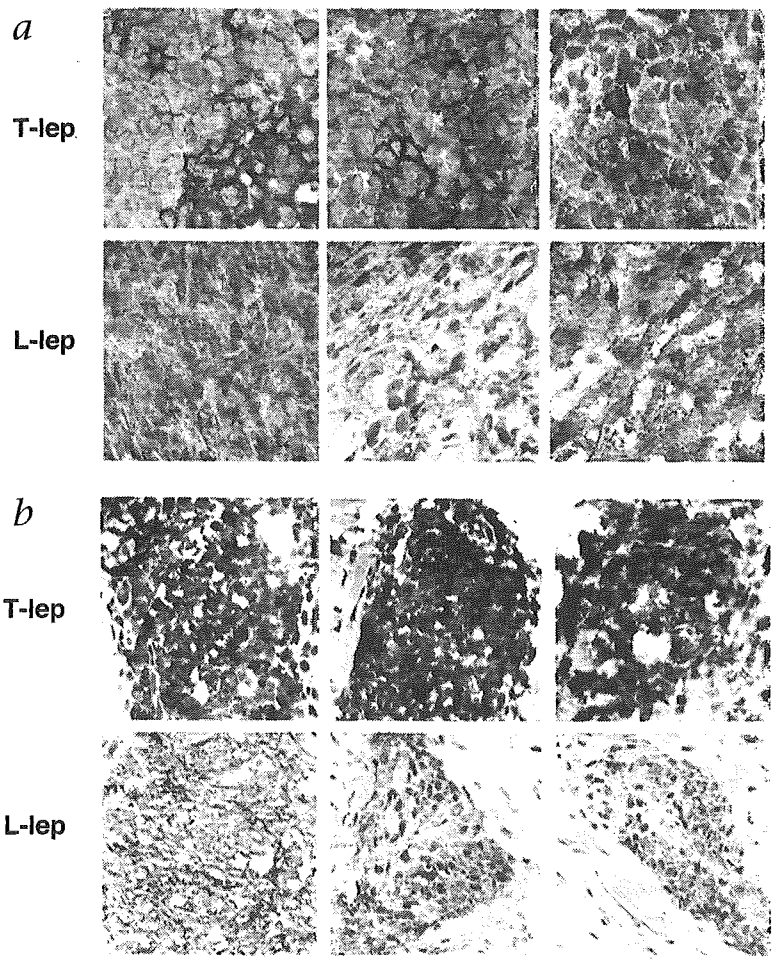

Fig. 5 Expression of TLR2 and TLR1 in leprosy patients. ***a*** and ***b***, Sections from skin biopsy specimens of T-lep ($n = 10$) and L-lep ($n = 10$) lesions stained by the immunoperoxidase method with a monoclonal antibody specific for TLR2 (*a*) or TLR1 (*b*). Original magnification, ×40.

TLR2-TLR1 activation in leprosy

As an initial step to determine whether the expression of TLRs in leprosy might correlate with the disease type, we measured the amount of TLR2 and TLR1 expression on peripheral monocytes. We found that the amounts of TLR2 on circulating monocytes was similar in both T-lep and L-lep patients, whereas the amounts of TLR1 were undetectable (Fig. 4*a* and data not shown). Although TLR2 was expressed equally in both patient groups, there could have been differences in the TLR2-TLR1–dependent activation pathways. We therefore examined the responses of peripheral monocytes and monocyte-derived dendritic cells from T-lep and L-lep patients to the 19-kD lipopeptide. The ability of the 19-kD lipopeptide to trigger IL-12 p40 release from monocytes was similar in T-lep and L-lep patients (Fig. 4*b*). These responses were TLR2-dependent, as the TLR2-specific antibody

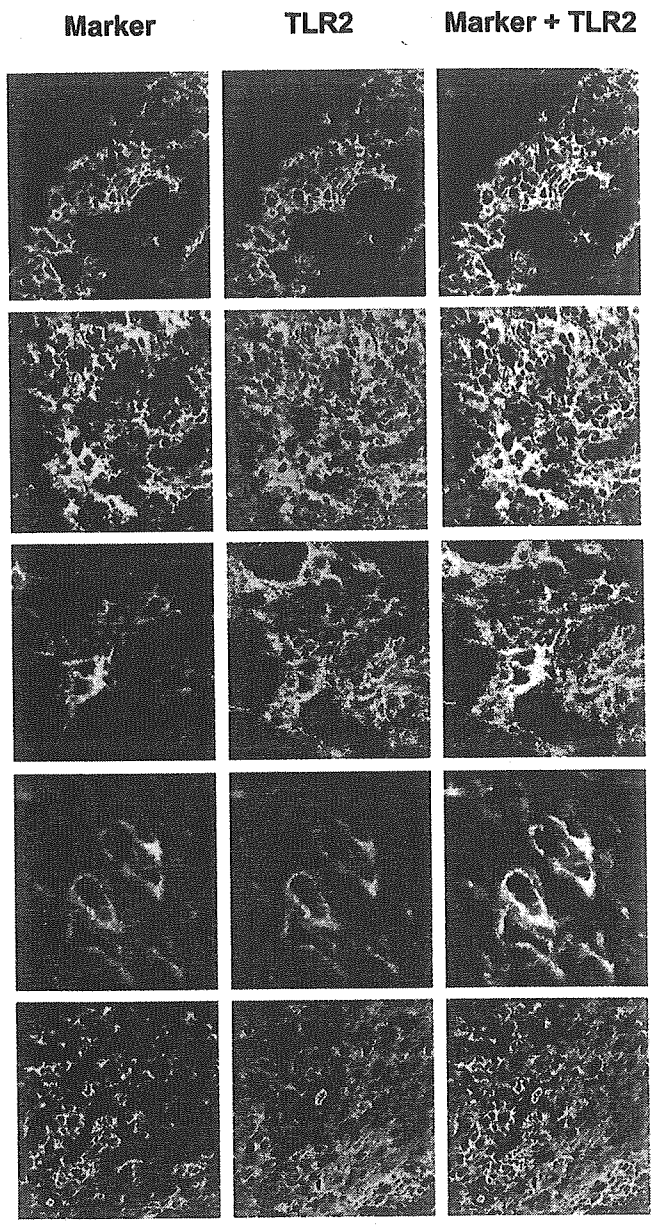

Fig. 6 Phenotype of cells expressing TLR2 in T-lep lesions. Two-color immunofluorescence confocal images were obtained for the markers CD14, CD68, CD1a, TLR1 or CD3 (green; left) and TLR2 (red; center). The 2 images were then superimposed (yellow; right). TLR2-positive cells in the T-lep lesions were positive for CD14, CD68, CD1a and TLR1, but not CD3. Original magnification, ×63.

blocked the response by 80% (data not shown). The 19-kD lipopeptide also induced similar levels of IL-12 p40 release from monocyte-derived dendritic cells from T-lep patients as compared with L-lep patients (Fig. 4*c*). These data indicate that in circulating blood, both T-lep and L-lep patients can mount a functionally equivalent TLR2-TLR1 response.

Expression of TLR2 and TLR1 in leprosy lesions

The battle between host immune response and microbial pathogens in leprosy is enjoined in the skin lesion, the site of disease activity. We wanted to determine whether the expression of TLR2 and TLR1 in the lesions of leprosy patients correlates with the local cytokine pattern characteristic of the different forms of the disease. We assessed the frequency and distribution of TLR2- and TLR1-expressing cells in biopsy specimens from patients with leprosy using immunoperoxidase labeling. Notable differences were observed at the level of both TLR2 and TLR1 expression. TLR2 was strongly expressed in T-lep lesions on large ovoid cells and on cells with a dendritic morphology (Fig. 5*a*). Of ten T-lep lesions studied, approximately 40–50% of the cells infiltrating the granulomas were positive for TLR2. In contrast, TLR2 was only weakly expressed on cells in ten L-lep lesions. Similarly, TLR1 was much more strongly expressed in T-lep lesions as compared with L-lep lesions (Fig. 5*b*).

We used double immune labeling and confocal laser microscopy to examine the nature of the cells expressing TLR2 and TLR1 in T-lep lesions (Fig. 6). Approximately 90–95% of TLR2-expressing cells expressed both CD14 and CD68, indicating expression of TLR2 on cells of the monocyte/macrophage lineage. An additional 5% of cells expressed both TLR2 and CD1a, indicating expression of TLR2 on dendritic cells in these lesions. Expression of TLR1 co-localized with TLR2, indicating that most cells expressed the TLR2-TLR1 heterodimer. Finally, TLR2-positive cells did not express the T-cell marker CD3. Together, these findings indicate that the frequency of monocytes and dendritic cells expressing TLR2 and TLR1 in leprosy lesions is higher in T-lep patients, the group with localized infection and expression of type-1 cytokines in lesions, as compared with L-lep patients, the group with disseminated infection and expression of type-2 cytokines in lesions.

Discussion

The discovery of mammalian TLRs has provided new insight into mechanisms of innate immunity to microbial pathogens. Activation of TLRs leads to the induction of antimicrobial pathways central to innate defense[15] as well as the upregulation of antigen presentation molecules and secretion of cytokines that influence the nature of the adaptive immune response[14]. Here, we undertook a comprehensive and integrated analysis of TLR responsiveness, regulation and distribution in human disease, by analyzing human leprosy. We focused on TLR2 and TLR1 because the bacteria that causes leprosy, *M. leprae*, was found to activate either TLR2 homodimers or TLR2-TLR1 heterodimers. In addition, scanning of the *M. leprae* genome revealed 31 lipoproteins that could serve as pathogen-associated molecular patterns to be recognized by the innate immune system. Synthetic lipopeptides representing putative 19-kD and 33-kD lipoproteins activated monocytes and monocyte-derived dendritic cells through TLR2. We found that expression and activation of TLR2 and TLR1 were regulated by type-1 and type-2 cytokine patterns. Analysis of lesions provided *in vivo* evidence that the local cytokine environment regulates the expression of TLR2 and TLR1, thus determining the outcome of the innate immune response against microbial infection.

Through our study of human leprosy, we correlated TLR2 and TLR1 expression in disease lesions with resistance and susceptibility to a human pathogen. Whereas TLR2 expression on peripheral monocytes of patients from across the spectrum of

leprosy was equivalent, we observed differences at the sites of disease in the skin lesions obtained from patients. TLR2 was strongly expressed on monocytes and dendritic cells in lesions from T-lep patients, which are self-healing and characterized by resistance to growth of *M. leprae*. In contrast, TLR2 was weakly expressed on monocytes in L-lep lesions, which are progressive and characterized by enormous quantities of the organism. Similarly, we found much higher TLR1 expression in T-lep lesions compared with L-lep lesions. Previous studies of leprosy lesions indicates that the local expression of the type-1 cytokines IFN-γ, IL-12, IL-18 and GM-CSF predominates in T-lep lesions, whereas the type-2 cytokines IL-4 and IL-10 characterize L-lep lesions[17,18,21,22]. Our data corroborate this by showing that IL-4 downregulates *TLR2* mRNA and protein expression on monocytes[20,23] and also downregulates TLR2 on dendritic cells. We hypothesize that the strong expression of the type-2 cytokine IL-4 in L-lep lesions accounts for the diminished expression of TLR2. In contrast, the type-1 cytokines IFN-γ and GM-CSF upregulate levels of TLR1 expression *in vitro*, suggesting that the high levels of these cytokines in T-lep lesions accounts for the higher levels of TLR1 expression. These findings indicate that in humans, the regulated expression of TLR2 and TLR1 contributes to the outcome between the host response and the microbial invader. These results also suggest that effects of the local cytokine environment on the expression of TLR2-TLR1 heterodimers is a possible mechanism by which some individuals are hyporesponsive to vaccination with the *Borrelia burgdorferi* OspA lipopeptide[4].

A notable finding of the present study was that type-1 and type-2 cytokine patterns differentially affected TLR2 and TLR1 activation by an *M. leprae* lipopeptide. The type-1 cytokines IFN-γ, GM-CSF, IL-12 and IL-18 enhanced TLR2 and TLR1 activation, whereas the type-2 cytokines IL-4 and IL-10 inhibited activation. It is noteworthy that IL-10 inhibited TLR2 and TLR1 activation but did not downregulate TLR2 or TLR1 expression as compared with IL-4, which inhibited both TLR2 expression and TLR2-TLR1 heterodimer activation. The type-1 cytokines IFN-γ, IL-12 and IL-18 upregulated TLR2-TLR1 heterodimer responses without affecting TLR2 expression. The type-1 cytokines IFN-γ and GM-CSF, however, increased TLR1 expression in monocytes and monocyte-derived dendritic cells, respectively. Thus, the local cytokine pattern triggered during microbial infection regulates the innate immune system through two different and independent mechanisms, one directly affecting TLR activation and the other modulating TLR expression. It is well known that the innate immune response serves an instructional role in shaping the adaptive immune response. Our data indicate that the adaptive immune response, by releasing cytokines from T cells that regulate TLR activation, can also influence the magnitude of the innate immune response.

The ability of the innate immune system to recognize and rapidly respond to microbial pathogens is crucial to the host defense against infection. The Toll family of receptors seems to be integral in this role, given that it has been conserved throughout hundreds of millions of years of evolution, and is found in both insects and mammals. In *Drosophila*, mutations in *Toll* or downstream signaling molecules substantially increase susceptibility to fungal and bacterial infections. Mice with spontaneous or targeted mutations in TLRs are more susceptible to bacterial infection, further implicating TLRs as critical receptors in mammalian host defense[24–29]. Recent studies also indicate that TLRs are required for optimal induction of innate immunity in mouse models of microbial infection[30,31]. Although genetic analysis of *TLR2* in L-lep patients detected a polymorphism in the cytoplasmic signaling domain of the TLR2 protein[32], we found that there was no difference in the ability of the TLR2-TLR1 ligand to trigger IL-12 p40 release from peripheral blood monocytes obtained from L-lep as compared with T-lep patients. Further experiments are warranted to determine whether such polymorphisms affect TLR activation pathways.

We found that *M. leprae* activates cells through TLR2 and TLR2-TLR1. Of the potential ligands that activate TLR2-TLR1, microbial lipoproteins have long been known to be potent immunostimulatory molecules. Experiments have shown that TLR2 mediates responsiveness to lipoproteins and lipopeptides[1,33]. Additional studies have shown that triacylated lipoproteins and lipopeptides activate a TLR2-TLR1 heterodimer[3,4]. Using a genome-wide scan, 31 putative lipoprotein genes were identified in the annotated genome sequence of *M. leprae*[34], together with 3 genes encoding enzyme systems required for processing the lipoprotein precursor and attaching the lipid group. All of these genes have orthologs in *M. tuberculosis*[35], which also contains about 60 additional genes for lipoproteins that have either been lost by the leprosy bacillus or retained in inactive form as pseudogenes. Among the latter group is *ML1966*, a gene orthologous to *lpqH* (Rv3763), which encodes the well-characterized 19-kD lipoprotein antigen of *M. tuberculosis*[36,37]. A synthetic *M. leprae* 19-kD lipopeptide induced IL-12 p40 release from human monocytes and monocyte-derived dendritic cells through TLR2. Similarly, a 33-kD lipoprotein known to be expressed by *M. leprae*[19] was studied and found to activate monocytes and monocyte-derived dendritic cells through TLR2. Because our data suggest a prominent role for TLR2 and TLR1 in mediating the response to *M. leprae*, we cannot rule out other TLR ligands such as lipoarabinomannan and peptidoglycan activating through TLR2 (refs. 38, 39). In addition, although the response through TLR4 was weak using the available killed *M. leprae*, previous studies suggest that live mycobacteria can activate TLR4 (ref. 38). Further investigation to identify the TLR4 ligand(s) in live *M. tuberculosis* should clarify this issue.

Our data suggest that the outcome of the immune response in leprosy is determined in part by the influence of the local cytokine environment on the regulated expression and activation of TLR2 and TLR1. Although TLR activation is a central part of innate immunity, TLR activation can also lead to immunopathology and tissue damage, as in the apoptosis and inflammation-mediated tissue injury seen in septic shock. Even in the T-lep form of leprosy, where the level of TLR activation is strong and the pathogen is contained, inflammation-mediated nerve injury is a prominent feature. It should be possible to design therapeutic agents that regulate TLR expression and activation. Although such interventions could be used as an adjuvant for immune responses for vaccines or to treat active infection, the potential consequence of tissue injury requires scrutiny.

Methods

TLR ligands. Killed *M. leprae* was obtained from P. Brennan (Colorado State University, Fort Collins, Colorado). *M. leprae* hexameric peptides with a tri-palmityl modification corresponding to 19-kD and 33-kD lipoproteins from *M. leprae* (Pam_3CSGGTQ and Pam_3CLPAWK, respectively) and peptides lacking the tri-palmityl modification were synthesized (Norgard and Bachem, Torrance, California). *Salmonella minnesota* LPS (Sigma, St. Louis, Missouri) was purified as described[40]. MALP-2 and Pam_3Cys-Ser-$(Lys)_4$ (CSK_4) were used as described[2,41].

Patients and clinical specimens. Patients with leprosy were classified according to the criteria of Ridley and Jopling[16]. Scalpel or punch skin biopsy specimens (6 mm diameter) were obtained after informed consent (Institutional Review Board, University of Southern California School of Medicine) from 10 T-lep and 10 L-lep leprosy patients at the time of diagnosis. Specimens were embedded in OCT medium (Ames, Elkhart, Indiana), snap-frozen in liquid nitrogen and stored at –70 °C until sectioning.

Antibodies and cytokines. We used antibodies against TLR2 (2392; Genentech, San Francisco, California), TLR1 (eBioscience, San Diego, California), CD14 (Zymed, San Francisco, California), CD1a (OKT6 IgG1; ATCC, Manassas, Virginia), CD3 (OKT3 IgG1; ATCC), CD68 (Y1/82A; BD Pharmingen, San Diego, California) and IgG controls (Sigma). The following cytokines were used: IFN-γ (Endogen, Woburn, Massachusetts), IL-4 (Peprotech, Rocky Hill, New Jersey), IL-10 (BD Pharmingen), IL-12 (BD Pharmingen), IL-18 (R&D Systems, Minneapolis, Minnesota) and GM-CSF (Immunex, Seattle, Washington).

Monocytes, dendritic cells and ELISA. Monocytes were isolated by adherence in 1% FCS as described[1]. For dendritic cells, adherent cells were cultured in RPMI with 1,000 U/ml IL-4 and 750 U/ml GM-CSF and matured for 7 d. Monocytes and dendritic cells were stimulated with medium, killed *M. leprae*, or *M. leprae* 19-kD or 33-kD lipopeptides in the presence or absence of IFN-γ (100 U/ml), IL-12 (100 ng/ml), IL-18 (100 ng/ml), GMCSF (800 U/ml), IL-4 (1,000 U/ml) or IL-10 (100 U/ml). Antibody blocking was performed as described[1]. IL-12 p40 or TNF-α production was measured by ELISA after 24 h (BD Pharmingen).

Bacterial genomics. During the annotation of the *M. leprae* genome sequence[34], lipoproteins were identified by the presence of an appropriately positioned PROSITE motif (PS00013 prokaryotic membrane lipoprotein lipid attachment site[42]) following a signal peptide, allowing no mismatches. Lipoprotein gene and protein sequences were obtained from the Leproma database (http://genolist.pasteur.fr/Leproma/).

Transfection and reporter assays. Human embryonic kidney 293 cells were transiently transfected as described[3]. Twenty-four hours after transfection, cells were stimulated with *M. leprae* for 8 h. Cells were lysed and luciferase activity was measured as described[3].

Mouse peritoneal macrophages and ELISA. *Tlr1*$^{-/-}$, *Tlr2*$^{-/-}$, and *Tlr6*$^{-/-}$ mice were generated as described[3,8,10]. Isolation of peritoneal macrophages was done as described[15]. Macrophages (10^5) were stimulated for 24 h. TNF-α (Genzyme Techne, Minneapolis, Minnesota) and IL-6 (R&D Systems) production were measured by ELISA.

Cell surface labeling. Surface expression of TLR1, TLR2, CD14 and CD1a was determined using marker-specific antibodies. TLR1 and TLR2 signals were amplified with a biotin-conjugated goat antibody against mouse IgG (Southern Biotechnology Associates, Birmingham, Alabama) and a streptavidin-phycoerythrin conjugate (Caltag, Burlingame, California). Cells were fixed with 1% paraformaldehyde, acquired on a Becton Dickinson FacsScan (Mountain View, California) and analyzed using WinMDI 2.8 (J. Trotter, Scripps Research Institute, San Diego, California).

Real-time quantitative PCR. Monocytes were stimulated for 12 h with medium or IL-4 (0.01–1,000 U/ml). RNA was isolated using Trizol Reagent (Invitrogen, Carlsbad, California). cDNA was synthesized using the SUPERSCRIPT Choice System Kit (Invitrogen). The following h36B4 primers and probes were designed using Primer Express (Applied Biosystems, Branchburg, New Jersey): 5′-CCACGCTGCTGAACATGCT-3′ (forward), 5′-TCGAACACCTGCTGGATGAC-3′ (reverse), 5′-AACATGTCCCCCTTCTCCTTTGGGCT-3′ (probe); TLR2 primers and probe sequences 5′-GGCCAGCAAATTACCTGTGTG-3′ (forward), 5′-AGGCGGACATCCTGAACCT-3′ (reverse), 5′-TCCATCCCATGTGCGTGGCC-3′ (probe). Reactions were carried out in TaqMan Universal PCR Master Mix (Applied Biosystems) under recommended conditions, run on the ABI PRISM 7700 and analyzed with Sequence Detector Software (Applied Biosystems). The relative quantities of TLR2 per sample were calculated using the ΔΔ CT formula as described[43]. The data were normalized by fold change to media control. The assay was repeated 3 times and values were averaged.

Immunoperoxidase labeling, immunofluorescence labeling and microscopy. Immunoperoxidase labeling of cryostat sections was done as described[44]. TLR1- and TLR2-positive cells in dermal granulomas were quantitated by calculating the percentage of positive cells based on the total number of cells within the granuloma, as described[44]. Double immunofluorescence was performed by serially incubating sections with antibodies against CD markers and TLR1, followed by incubation with isotype-specific fluorochrome (Caltag, Burlingame, California). Sections were washed with PBS containing 2% goat serum, incubated with TLR2-specific antibody for 1 h, then incubated with a tetramethyl rhodamine isothiocyanate (TRITC)–conjugated antibody against mouse IgG1 (Southern Biotechnology). Sections were washed and mounted in Vectashield medium (Vector laboratories, Burlingame, California). Controls were performed as described[45,46]. Double immunofluorescence was examined with a Leica-TCS-SP inverted confocal laser scanning microscope as described[46].

Note: Supplementary information is available on the Nature Medicine *website.*

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Competing interests statement

The authors declare that they have no competing financial interests.

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