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Figure legends

Figure 1. Construction of pSO246mIL-15 (*A*) and detection of Ag85B-IL15 from the lysate of rBCG-Ag85B-IL15 by Western blotting (*B*). The pellet of rBCG-Ag85B-IL15, rBCG-Ag85B or BCG-plasmid-vector were suspended in lysis buffer for 30 min or the bacteria were cultured in 7H9 medium for 1 week, and after the centrifugation, supernatants were harvested. After the protein quantitation of the supernatants, samples applied to SDS-PAGE, and then were blotted and reacted with anti-IL15 Ab or anti-Ag85B serum.

Figure 2. Bacterial growth in different organs of C57BL/6 mice after immunization with rBCG-Ag85B-IL15. C57BL/6 mice were i.p. immunized with 5×10^6 CFU of rBCG-Ag85B-IL15 and rBCG-Ag85B. The numbers of bacteria recovered from the spleen, peritoneal cavity or lung of immunized mice were determined on indicated days. "→" shows the inoculation doses of the rBCG strains that were checked just after the i.p. injection. Data of a representative are shown from three separate experiments and are expressed as means ± SD of four mice of each group. *, $p<0.05$, **, $p<0.01$.

Figure 3. Kinetics of absolute numbers of T cell subsets in mice after rBCG-Ag85B-IL15 immunization. The cells of the spleen, PEC and lung on day 0, 7, 21, 42 or 70 after rBCG-Ag85B-IL15 or rBCG-Ag85B immunization were stained with various monoclonal Abs, and the absolute numbers of $CD44^+CD8^+$ and $CD44^+CD4^+$ T cells were calculated by multiplying total splenocytes, PECs or lung MNCs by the percentages of each subset in the spleen, PEC or lung. Data

of a representative are shown from three separate experiments and are expressed as means ± SD of three mice of each group. *, $p<0.05$, **, $p<0.01$.

Figure 4. IFN-γ production by Ag-stimulated T cells in the spleen, PEC and lung of mice after rBCG-Ag85B-IL15 immunization. Intracellular expression of IFN-γ by $CD8^+$ and $CD4^+$ T cells from C57BL/6 mice on day 21 after immunization with rBCG-Ag85B-IL15 and rBCG-Ag85B. Cells were cultured with PPD (*A*) or MPT-64, TB2 or Peptide-25 (*B*) and surface-stained with anti-CD8, -CD4 and -CD44 mAbs, and intracellular-stained with anti-IFN-γ mAb. Intracellular cytokine-producing cells were examined using a flow cytometer and were analyzed by gating on $CD8^+$ or $CD4^+$ T cells. The data are representative of two separate experiments and are expressed as means ± SD of three mice of each group. *, $p<0.05$

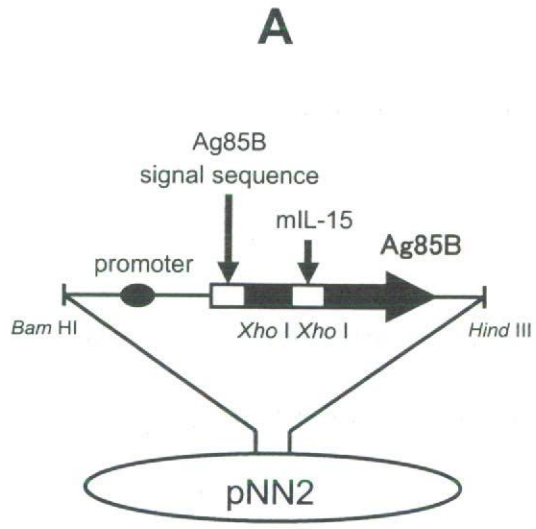
Figure 5. IFN-γ production by Ag-stimulated T cells in the spleen, PEC and lung of mice after rBCG-Ag85B-IL15 immunization. Intracellular expression of IFN-γ by $CD8^+$ and $CD4^+$ T cells from C57BL/6 mice on day 70 after immunization with rBCG-Ag85B-IL15 and rBCG-Ag85B. Cells were cultured with PPD, TB2 or Peptide-25 (*A*) and surface-stained with anti-CD8, -CD4 and -CD44 mAbs, and intracellular-stained with anti-IFN-γ mAb. Intracellular cytokine-producing cells were examined using a flow cytometer and were analyzed by gating on $CD8^+$ or $CD4^+$ T cells. *B.* $CD8^+$ or $CD4^+$ T cells on day 70 after rBCG-Ag85B-IL15 or rBCG-Ag85B immunization were purified and cultured with TB2 or Peptide-25, and IFN-γ production in the supernatants were assayed by ELISA.

The data are representative of two separate experiments and are expressed as means ± SD of three mice of each group. *, $p<0.05$, **, $p<0.01$

Figure 6. Mycobacterial growth and histologic examination of lung tissues from rBCG-Ag85B-IL15-immunized mice after an intratracheal infection with *M. tuberculosis* H37Rv. On day 90 after the inoculation, mice were treated with isoniazid in drinking water for 4 weeks to clear the live BCG bacteria and then on day 127 rmice were i.t. infected with 2×10^5 CFU of *M. tuberculosis* H37Rv. Mice were sacrificed 10 weeks after the infection, and the numbers of bacteria recovered from lungs and spleens were determined (*A*). The data are representative of two separate experiments and are expressed as means ± SD of five mice of each group. *, $p<0.05$, **, $p<0.01$. Formalin-fixed sections were stained with hematoxylin and eosin (*B*). Magnification, ×50 or ×200. An example from one of two separate experiments is shown.

Figure 1

Figure 1. Tang *et al.*



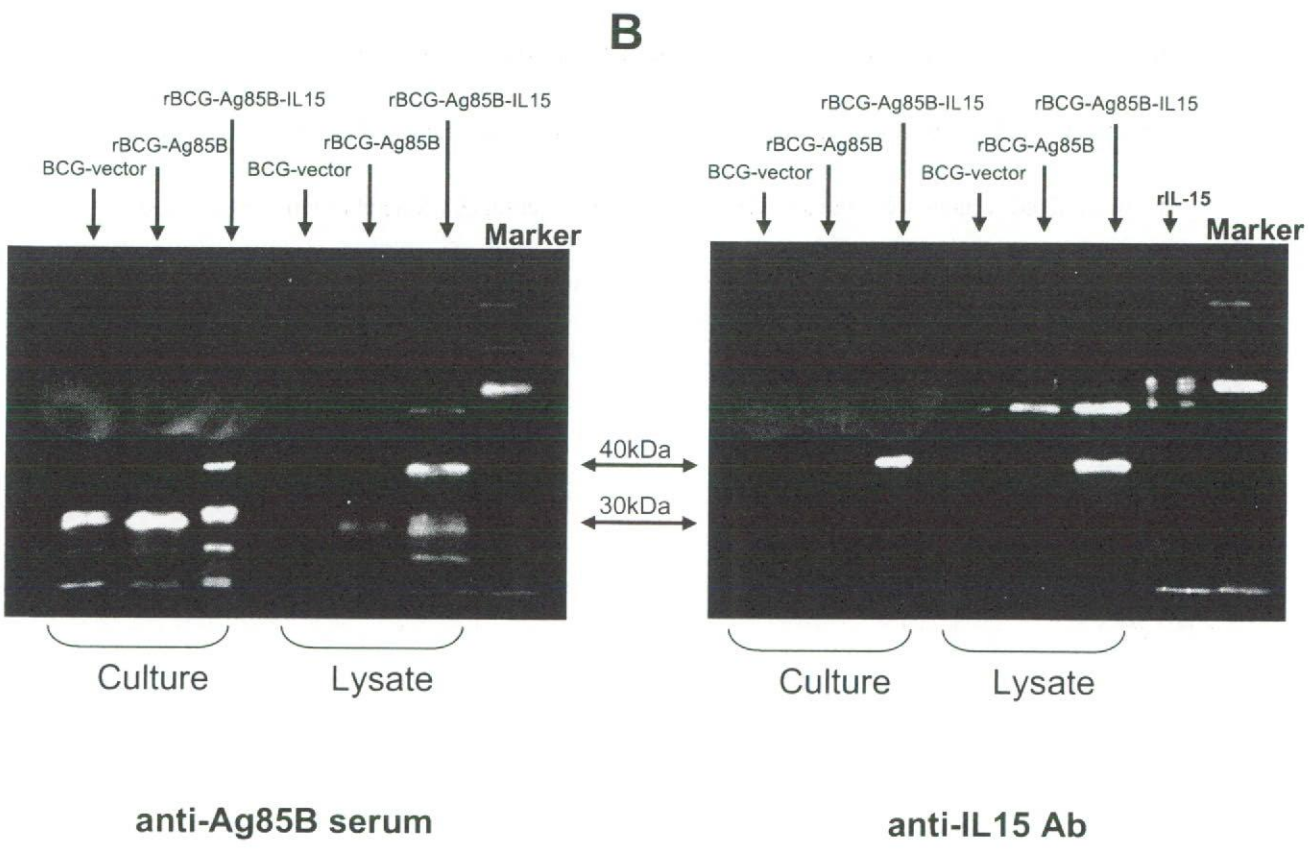


Figure 2

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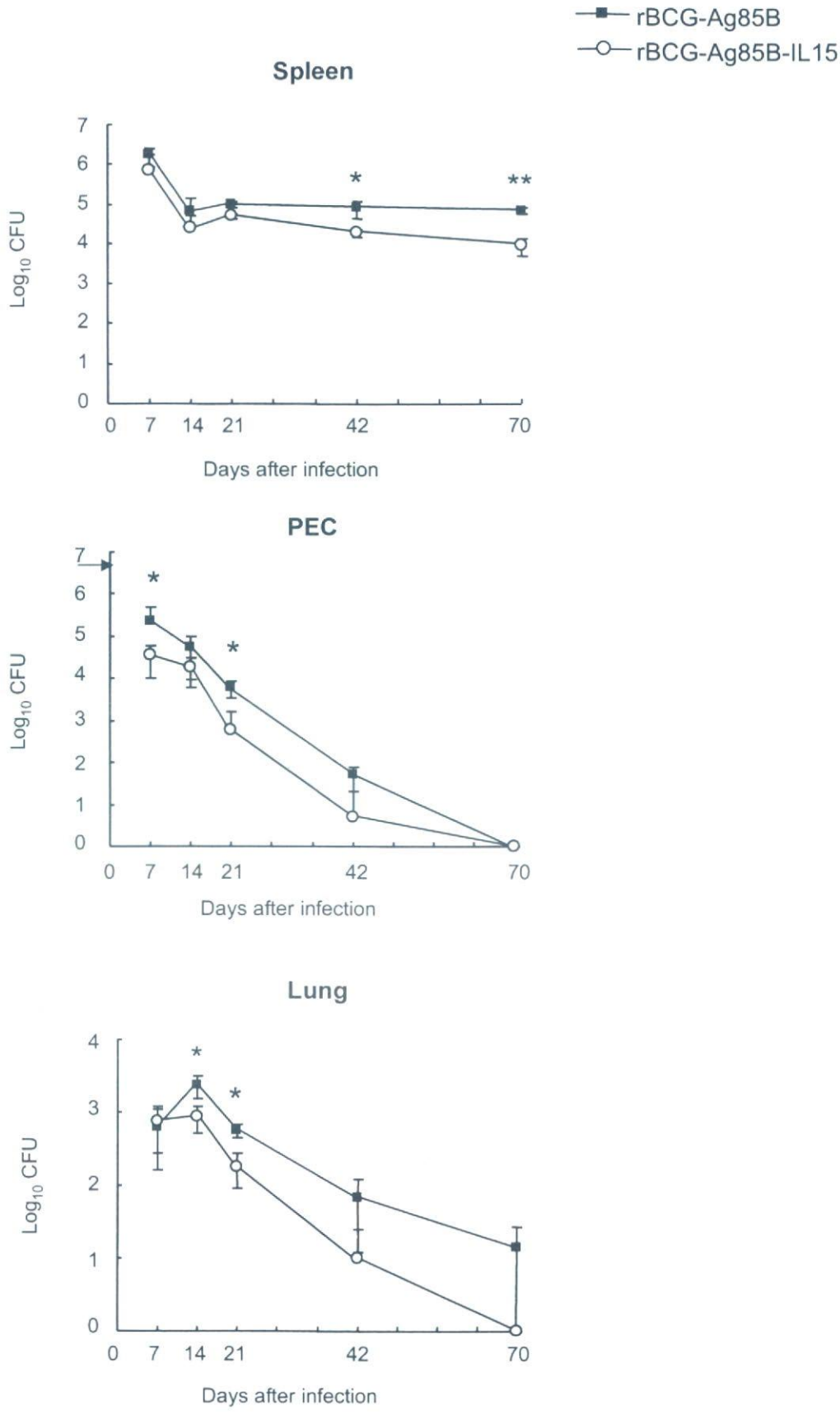

Figure 3

Figure 3. Tang *et al.*

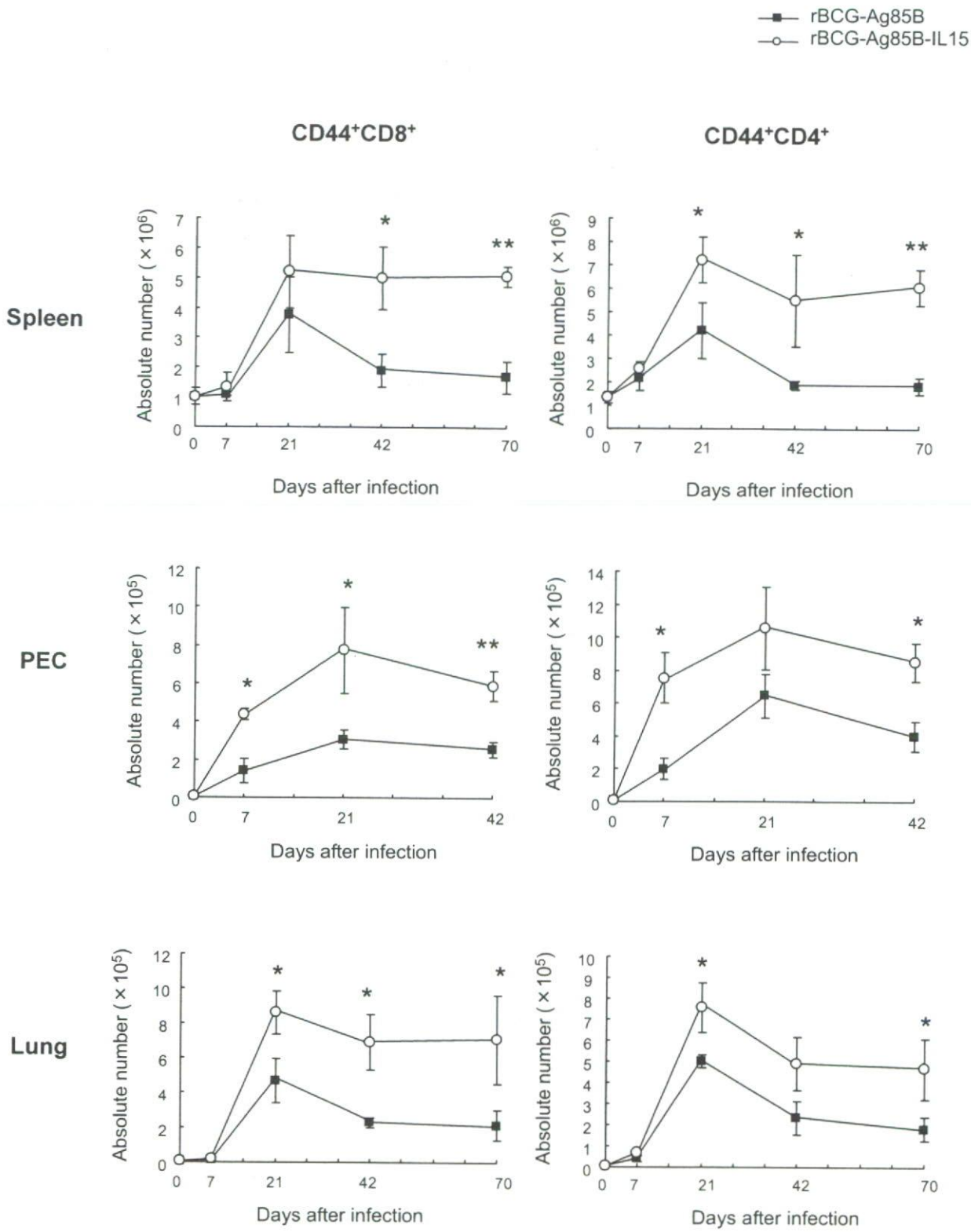

Figure 4

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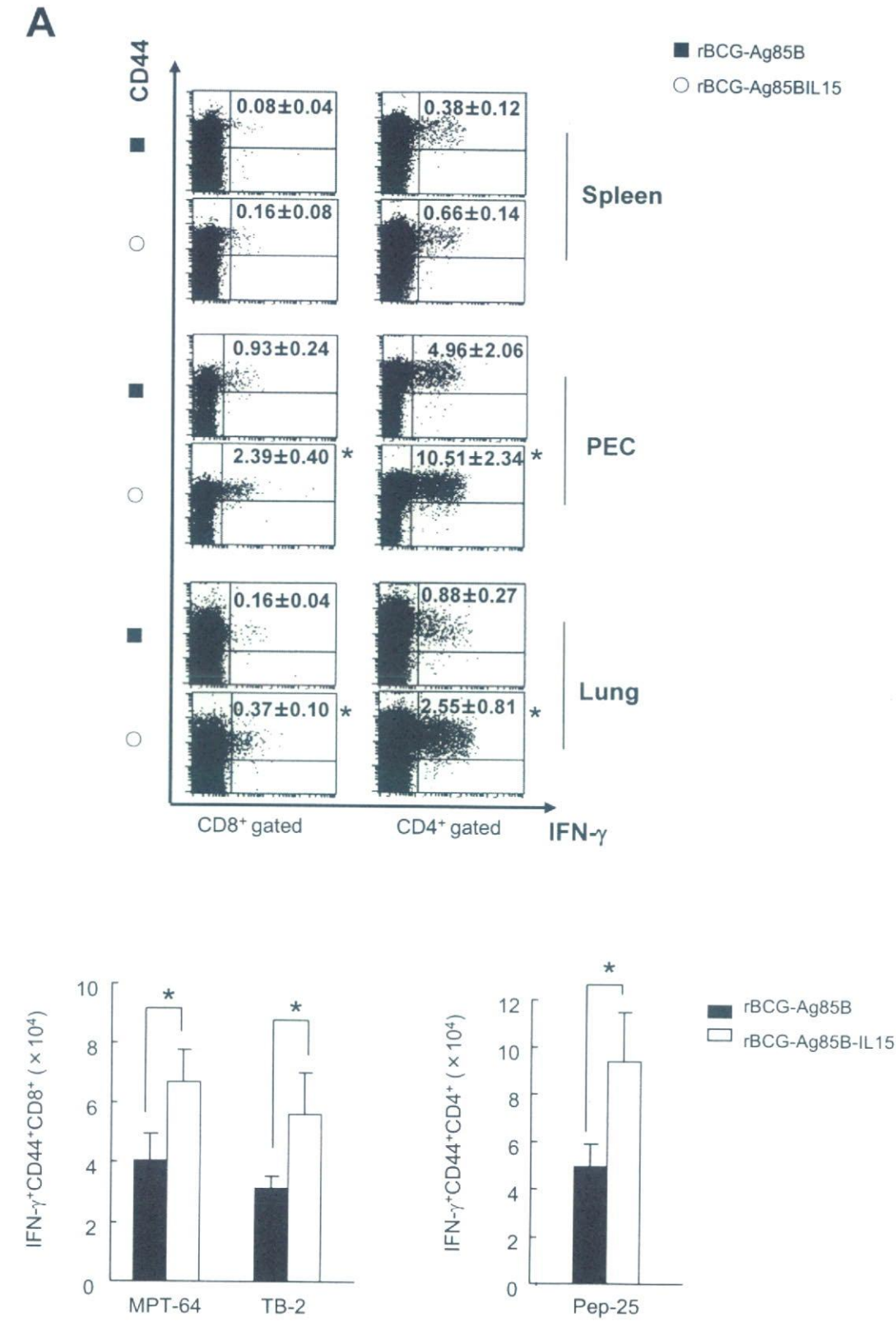

Figure 5

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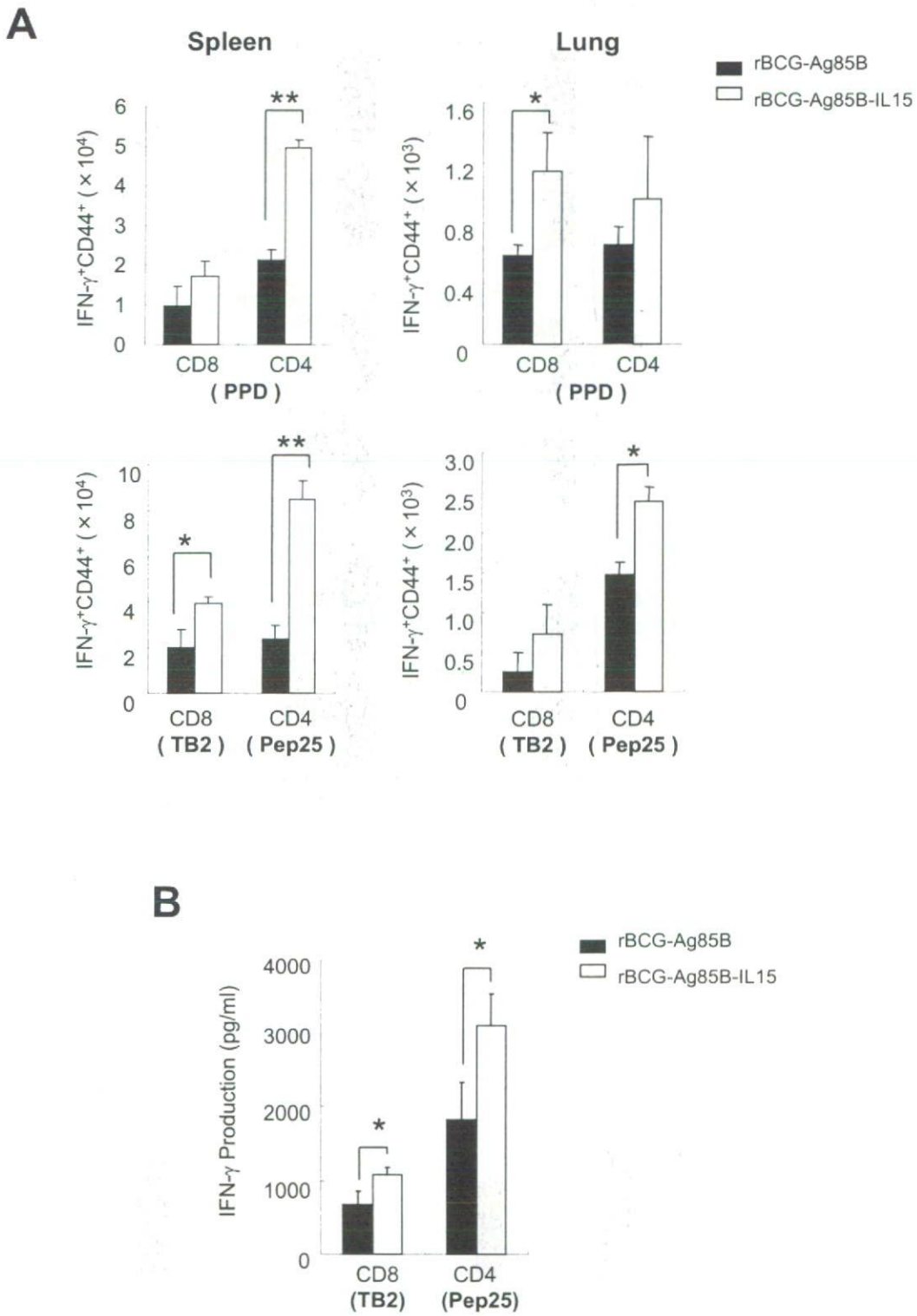

Figure 6

Figure 6. Tang *et al.*

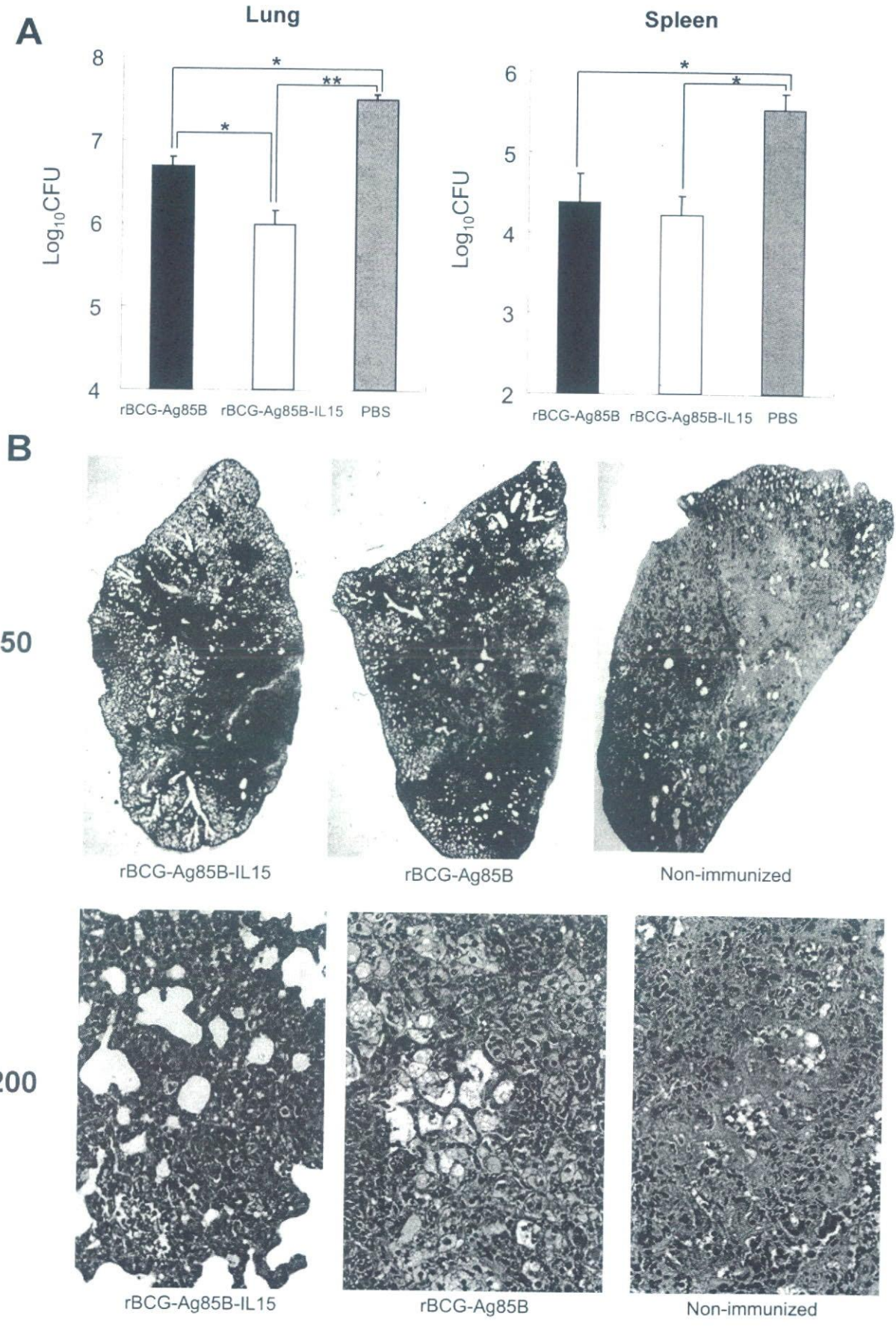

Table 1

Intracellular expression of IFN-γ by T cells in the lung or spleen from mice after intratracheal infection with *M. tuberculosis*

	$CD8^+$ T cells			$CD4^+$ T cells		
	% of $CD44^+IFN\text{-}\gamma^+$ in **CD8**		% of $CD44^+CD62L^+$ in **IFN-γ^+CD8**	% of $CD44^+IFN\text{-}\gamma^+$ in **CD4**		% of $CD44^+CD62L^+$ in **IFN-γ^+CD4**
Stimulation	Lung	Spleen	Spleen	Lung	Spleen	Spleen
PPD						
PBS	0.16±0.07	0.12±0.04		1.15±0.28	0.71±0.16	
rBCG-Ag85B	0.23±0.05	0.18±0.90		0.63±0.11	1.04±0.33	
rBCG-Ag85B-IL15	0.41±0.08 *	0.35±0.07		1.25±0.47 *	1.94±0.19 *	
TB-2						
PBS	0.11±0.04	0.63±0.11	22.19±6.44			
rBCG-Ag85B	0.11±0.03	0.57±0.09	38.90±5.43			
rBCG-Ag85B-IL15	0.26±0.04 *	0.97±0.44	65.56±12.21 *			
MPT-64						
PBS	0.11±0.03	0.41±0.17	37.86±6.39			
rBCG-Ag85B	0.12±0.05	0.37±0.12	44.08±6.25			
rBCG-Ag85B-IL15	0.18±0.03	0.81±0.16 *	65.06±8.97 *			
Peptide25						
PBS				0.59±0.19	0.68±0.20	27.33±8.08
rBCG-Ag85B				0.44±0.05	0.38±0.11	35.12±6.71
rBCG-Ag85B-IL15				0.84±0.21 *	0.73±0.19	53.24±8.35 *

NOTE- C57BL/6 mice were inoculated with rBCG-Ag85B-IL15, rBCG-Ag85B or PBS and on day 90 after the inoculation, mice were treated with isoniazid in drinking water for 4 weeks to clear the live BCG bacteria and then on day 127 mice were i.t. infected with 2×10^5 CFU of *M. tuberculosis* H37Rv. Lung MNCs or splenocytes were harvested from mice of each group 4 weeks after the *M. tuberculosis* infection and cultured with PPD, TB-2, MPT-64 or Peptide25. After culture, cells were stained with anti-CD8 / anti-CD4, anti-CD44 and anti-CD62L mAbs and then intracellular stained with anti-IFN-γ mAb. Intracellular cytokine-producing cells were examined and were analyzed by gating on $CD8^+$ / $CD4^+$ T cells or IFN-γ^+CD8^+ / IFN-γ^+CD4^+ T cells. Each number indicates mean percentage of $CD44^+$IFN-γ^+ cells in $CD8^+$ / $CD4^+$ T cells or $CD44^+CD62L^+$ cells in IFN-γ^+CD8^+ / IFN-γ^+CD4^+ T cells ± SD of three mice of each group. Data of a representative are shown from two separate experiments.

*, $p<0.05$, significantly different from the value for rBCG-Ag85B-immunized mice.

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Microbes and Infection 9 (2007) 70–77

www.elsevier.com/locate/micinf

Original article

Contribution of GM-CSF on the enhancement of the T cell-stimulating activity of macrophages

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Received 9 November 2005; accepted 10 October 2006
Available online 11 December 2006

Abstract

Mycobacterium leprae is an intracellular parasitic organism that multiplies in macrophages (MØ). It inhibits the fusion of mycobacterial phagosome with lysosome and induces interleukin (IL)-10 production from macrophages. However, macrophages are heterogenous in various aspects. We examined macrophages that differentiated from monocytes using either recombinant (r) granulocyte-MØ colony-stimulating factor (GM-CSF) (these MØ are named as GM-MØ) or rMØ colony-stimulating factor (M-CSF) (cells named as M-MØ) in terms of the T cell-stimulating activity. Although both macrophages phagocytosed the mycobacteria equally, GM-MØ infected with *M. leprae* and subsequently treated with IFN-γ- and CD40 ligand (L) stimulated T cells to produce interferon-gamma (IFN-γ), but M-MØ lacked the ability to stimulate T cells. While M-MØ mounted a massive IL-10 production, GM-MØ did not produce the cytokine on infection with *M. leprae*. *M. leprae*-infected, IFN-γ- and CD40L-treated GM-MØ expressed a higher level of HLA-DR and CD86 Ags than those of M-MØ, and expressed one of the dominant antigenic molecules of *M. leprae*, Major Membrane Protein-II on their surface. These results indicate that GM-CSF, but not M-CSF, contributes to the up-regulation of the T cell-stimulating activity of *M. leprae*-infected macrophages.

Keywords: Macrophage; *M. leprae*; GM-CSF; IFN-γ

1. Introduction

Mycobacterium leprae (*M. leprae*), a causative agent of human leprosy, is a representative parasitic pathogen that induces skin lesions and chronic progressive peripheral nerve injury, leading to systemic deformity [1,2]. Leprosy represents a clinical spectrum, in which clinical manifestations are associated with different levels of immune responses to *M. leprae* infection [3]. One representative type is a tuberculoid leprosy, in which patients exhibit innate and adaptive immunities to *M. leprae* and manifest a localized form of the disease with granuloma formation in infected tissues [4–6]. For the activation of an adaptive immunity, dendritic cells (DC) derived from inflammatory monocytes, play a central role [7,8]; and, in *in vitro* experiments, both $CD4^+$ and $CD8^+$ T cells are activated by DC infected with *M. leprae*, and these DC expressed Major Membrane Protein-II (MMP-II) as a dominant antigenic molecule [9,10]. Another representative manifestation is lepromatous leprosy, in which patients show reduced levels of host defense associated immunities and manifest a disseminated form of the disease with a broad spread of foamy MØ, in which an abundance of bacilli are usually involved [11,12]. *M. leprae* resides in the phagosome in MØ and replicates there without being digested by lysosomal enzymes [13]. Furthermore, *M. leprae* stimulates MØ to produce IL-10 [5,6] and suppresses the DC-mediated Ag-specific

Abbreviations: Ag, antigen; APC, Ag-presenting cells; BCG, *Mycobacterium bovis* BCG; DC, dendritic cells; GFP-BCG, BCG expressing GFP; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN-γ, interferon-gamma; IL, interleukin; L, ligand; mAb, monoclonal antibody; MØ, macrophages; M-CSF, MØ colony-stimulating factor; *M. leprae*, *Mycobacterium leprae*; MMP-II, Major Membrane Protein-II; PBMC, peripheral blood mononuclear cells; r, recombinant.

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doi:10.1016/j.micinf.2006.10.011

adaptive immunity [14,15]. These observations may indicate that the induction of intracellular processing of *M. leprae* and that of expression of molecules, such as MMP-II could lead to the activation of IFN-γ producing type 1 $CD4^+$ T cells. Another important element that should be considered for the full activation of T cells is the suppression of IL-10 production from *M. leprae*-infected MØ.

So far, a variety of methods and tools, including cytokines, have been used for the differentiation of MØ from human peripheral monocytes *in vitro* [16–18]. One representative MØ can be differentiated by using M-CSF, termed M-MØ, and another by using GM-CSF, termed GM-MØ. Both MØ represent different functions on infection with mycobacteria. However, much remains not fully understood with regard to *M. leprae* infection and the T cell-stimulating activity of these MØ.

In this report, we analyzed the characteristics of *M. leprae*-infected GM-MØ and M-MØ, and tried to develop immunological methods to enhance the MØ-mediated host defense activities against the bacteria.

2. Materials and methods

2.1. Preparation of cells and bacteria

Peripheral blood was obtained from healthy PPD-positive individuals under informed consent. We are aware that PPD-negative individuals would help to provide more information for our study; however, in Japan, most healthy individuals are PPD-positive, because *Mycobacterium bovis* BCG vaccination is compulsory for children (0–4 years old). Moreover, PPD-negative individuals in the Japanese population are those who do not respond to BCG vaccination, and therefore, it is likely that they suffer from some immune insufficiency. Therefore, these individuals cannot be used as controls for our experiments. Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Paque Plus (Pharmacia, Uppsala, Sweden) and cryopreserved in liquid nitrogen until use, as previously described [19]. 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 monoclonal antibody (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 [19]. MØ were differentiated by culturing monocytes in the presence of 20% fetal calf serum and either rM-CSF (R and D Systems, Abingdon, UK) (M-MØ) or rGM-CSF (Pepro Tech EC LTD, London, UK) (GM-MØ) [20]. Both GM-MØ and M-MØ were pulsed with *M. leprae*, treated with an optimal dose of IFN-γ on day 3 of culture, further treated with CD40L on day 4, and were used as a stimulator of T cells on day 5 [21]. *M. leprae* (Thai-53) was isolated from the footpads of BALB/c-*nu/nu* mice [22]. The isolated bacteria were counted by Shepard's method [22]. Killed *M. leprae* was prepared by heating the bacteria at 60 °C for 18 h. 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. The GFP sequence was amplified from pEGFP-1 vector (CLONTECH, Palo Alto, CA), and cloned into pMV261 [23]. Transformants were selected on a 7H10 plate containing 25 μg/ml kanamycin. The phagocytosis of BCG by GM-MØ and M-MØ after culture was determined using FACScalibur (Becton Dickinson Immunocytometry System, San Jose, CA). The multiplicity of infection (MOI) was determined based on the assumption that MØ were equally susceptible to infection with *M. leprae* [24].

2.2. Analysis of cell surface antigen (Ag)

The expression of cell surface Ag on MØ was analyzed using FACScalibur. Dead cells were eliminated from the analysis by staining with propidium iodide (Sigma Chemical Co., St. Louis, MO) and 1×10^4 live cells were analyzed. For analysis of cell surface Ag, the following mAbs were used: FITC-conjugated mAb against HLA-ABC (G46-2.6, PharMingen, San Diego, CA), HLA-DR (L243, PharMingen), CD14 (M5E2, BD Biosciences, San Jose, CA), TLR2 (TL2.3, Serotec, Oxford, UK), TLR4 (HTA125, Santa Cruz Biotech, Santa Cruz, CA), CD209 (DCN46, PharMingen), CD86 (FUN-1, PharMingen), and CD40 (5C3, PharMingen).

The expression of MMP-II, which is one of the dominant antigenic molecules of *M. leprae* [9] on *M. leprae*-infected MØ was determined using the mAb (IgM, kappa) against MMP-II, followed by FITC-conjugated anti-mouse Igs Ab (Tago-immunologicals, Camarillo, CA).

2.3. APC function of M. leprae-infected MØ

The ability of *M. leprae*-infected MØ to stimulate T cells was assessed using an autologous MØ-T cell co-culture as previously described [24,25]. Freshly thawed PBMC were depleted of $CD56^+$, MHC class II^+ and $CD8^+$ cells by using magnetic beads coated with mAb to CD56, MHC class II and CD8 Ags (Dynabeads 450; Dynal) [25]. The purity of $CD4^+$ T cells was more than 98% as assessed by FACS analyses. The purified responder cells (1×10^5 per well) were plated in 96-well round-bottom tissue culture plates and MØ were added to give the indicated MØ:$CD4^+$ T cell ratio. Supernatants of MØ-T cell co-cultures were collected on day 4 and the concentration of cytokines was determined.

2.4. Cytokine production

Levels of the following cytokines were measured; IFN-γ produced by $CD4^+$ T cells, IL-10, IL-1β, TNFα and IL-12p40 produced by MØ stimulated for 24 h with *M. leprae*. The concentrations of these cytokines were quantified using the enzyme assay kits, Opt EIA Human ELISA Set (BD PharMingen International).

2.5. Statistical analysis

Student's *t*-test was applied to determine the statistical differences.

3. Results

3.1. Characteristics of MØ differentiated from monocytes using GM-CSF

MØ were differentiated from monocytes using either GM-CSF (GM-MØ) or M-CSF (M-MØ). To characterize these two types of MØ, surface markers expressed on GM-MØ and M-MØ were analyzed using the monocytes obtained from the same donor by flow cytometry (Fig. 1). MHC class I (HLA-ABC) and class II (HLA-DR) Ags were similarly expressed on GM-MØ and M-MØ, but the expression of CD14 Ag was significantly reduced in GM-MØ. While the expression level of TLR2, CD209, CD40 and TLR4 Ags was not different between GM-MØ and M-MØ, the expression of CD86 was significantly higher on GM-MØ than M-MØ. Then, we examined the phagocytic capacity of GM-MØ and M-MØ by using BCG expressing GFP (GFP-BCG), since *M. leprae* cannot be cultured *in vitro* or express GFP. The percentage of MØ expressing GFP after co-culture of MØ with GFP-BCG was similar between GM-MØ and M-MØ (Fig. 2). These results indicate that GM-MØ and M-MØ differed in the expression of some surface markers, but they similarly phagocytosed the mycobacteria.

3.2. Effect of M. leprae infection to GM-MØ on the T cell-stimulating activity

Since *M. leprae* is an intracellular parasitic bacterium and is hardly digested with lysosomal enzyme in MØ unless MØ are activated [26], we analyzed the T cell-stimulating activity of *M. leprae*-infected GM-MØ and M-MØ (Table 1). When M-MØ were infected with up to MOI 80 of *M. leprae* and treated with IFN-γ and CD40L, they did not stimulate $CD4^+$ T cells to secrete a significant dose of IFN-γ. In contrast to M-MØ, when *M. leprae*-infected, IFN-γ- and CD40L-treated GM-MØ were used as Ag-presenting cells (APC), T cells produced significant levels of IFN-γ in a manner dependent on the dose of *M. leprae*. Since GM-MØ express CD40, and are activated by IFN-γ, we examined the effect of treatment with IFN-γ and CD40L on the T cell-stimulating activity of *M. leprae*-infected GM-MØ (Table 2). While IFN-γ production from $CD4^+$ T cells was not significantly induced by GM-MØ untreated or treated with either IFN-γ or CD40L, the cytokine production was significantly enhanced by the treatment of GM-MØ with

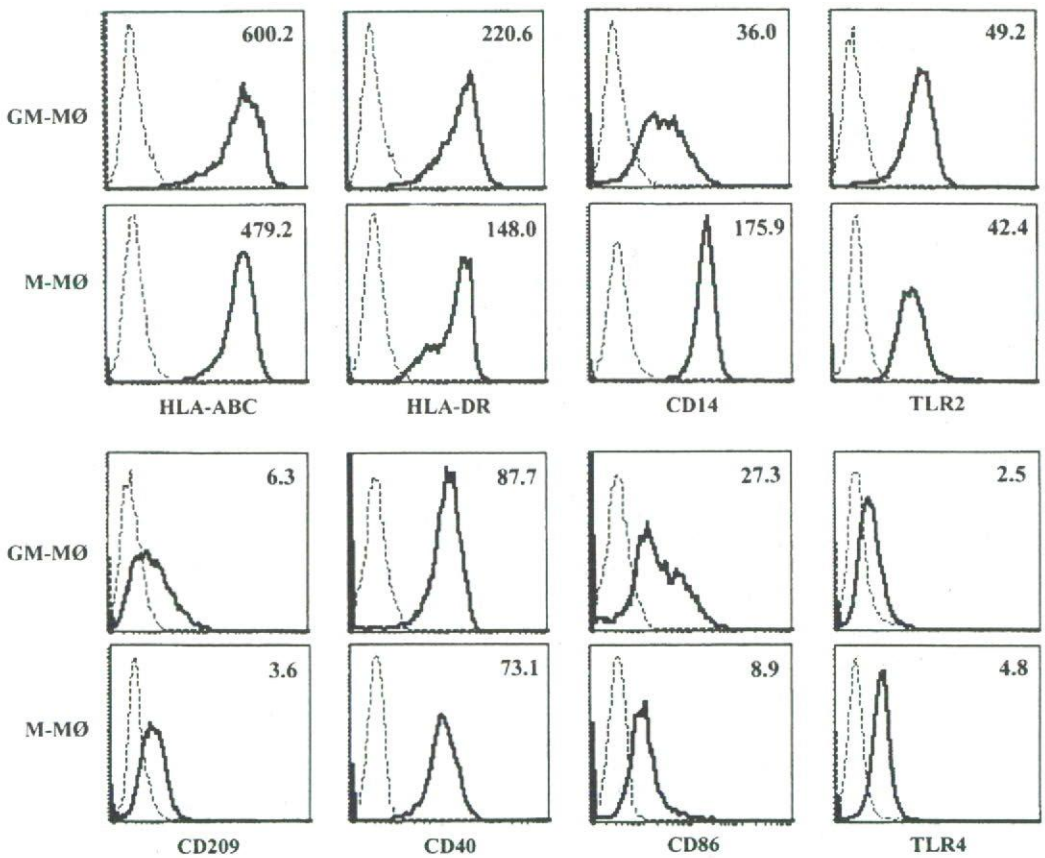

Fig. 1. Phenotype of GM-MØ and M-MØ differentiated from monocytes. Plastic adherent monocytes were differentiated into MØ by 3 days culture with either rGM-CSF or rM-CSF. Dashed lines, isotype-matched control IgG; solid lines, mAb staining. The number represents the difference in mean fluorescence intensity between dashed and solid lines. Representatives of three independent experiments are shown.

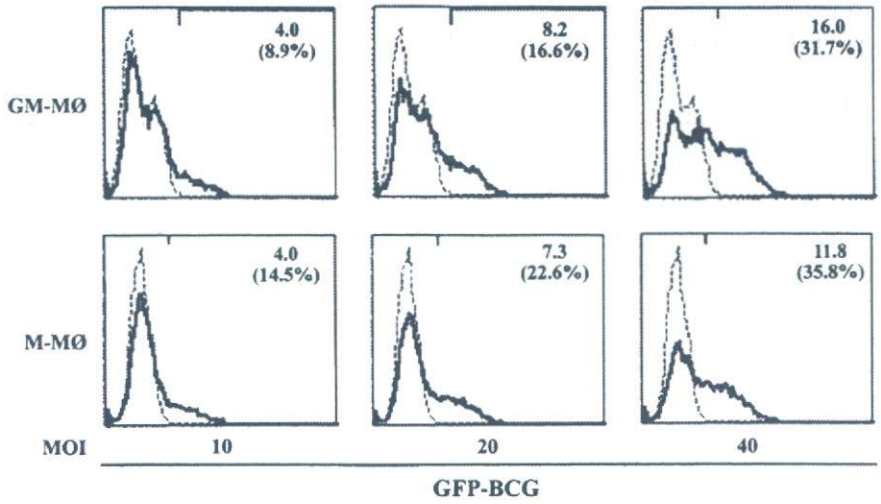

Fig. 2. Phagocytic activity of GM-MØ and M-MØ. Plastic adherent monocytes were differentiated into MØ by 3 days culture with either rGM-CSF or rM-CSF. For analysis of the phagocytic activity of MØ, GM-MØ and M-MØ were pulsed with BCG expressing GFP and assessed on day 4 of culture. Dashed lines, unpulsed cells; solid lines, GFP-BCG pulsed cells. The number represents the difference in mean fluorescence intensity between the dashed and solid lines. The number in parenthesis indicates the percent GFP-positive cell number. Representatives of three independent experiments are shown.

both IFN-γ and CD40L. Then, we compared the T cell-stimulating activity of live and heat-killed *M. leprae* (Table 3). Both forms of *M. leprae* stimulated $CD4^+$ T cells when pulsed to GM-MØ, but the heat-killed *M. leprae* more efficiently induced T cell activation than live bacteria. When we examined the effect of heat-killed *M. leprae* on M-MØ, they did not stimulate $CD4^+$ T cells significantly, even when IFN-γ and CD40L were administered (data not shown). Also note that, when GM-MØ and monocyte-derived DC were compared in terms of their T cell-stimulating activity, GM-MØ were less efficient in this respect (data not shown).

3.3. Factors associated with the enhancement of the T cell-stimulating activity of GM-MØ

Various factors may be responsible for enhancing the T cell-stimulating activity of APC. When we examined the expression of APC associated molecules on *M. leprae*-infected MØ (Fig. 3), the expression of HLA-DR and CD86 on GM-MØ was higher than on M-MØ, although there was no significant difference in the expression of HLA-ABC between GM-MØ and M-MØ. The cytokines produced from APC should also be considered to be another important factor that should be monitored and MØ produce a variety of cytokines, including IL-10, IL-1β, TNFα and IL-12 [6,11,27]. IL-10 was efficiently produced from M-MØ by stimulation with *M. leprae*, but it was hardly produced from GM-MØ (Fig. 4a). When macrophages were differentiated by using both GM-CSF and M-CSF, the function of GM-CSF was dominant and, the production of IL-10 was suppressed (Fig. 4a). Similarly to the production of IL-10, IL-1β was more efficiently produced from M-MØ than GM-MØ (Fig. 4b). In contrast, TNFα, which is important for granuloma formation, was more efficiently produced from GM-MØ (Fig. 4c). However, there was no significant difference in the production of IL-12p40 between GM-MØ and M-MØ (Fig. 4d). Finally, we assessed whether *M. leprae*-infected GM-MØ expressed dominant antigenic molecules of *M. leprae* on the surface (Fig. 5). To this end, we examined the expression of MMP-II on GM-MØ and M-MØ. No apparent expression of

Table 1
T cell-stimulating activity of *M. leprae*-infected GM-MØ and M-MØ[a]

Stimulator of $CD4^+$ T cells	*M. leprae* infection of macrophages (MOI)	IFN-γ (pg/ml) production by $CD4^+$ T cells after stimulation with macrophages at ratio (T:MØ)		
		2:1	4:1	8:1
GM-MØ	0	$0.6 \pm 0.2^{*,\dagger}$	$0.5 \pm 0.1^{\ddagger,\S}$	$1.4 \pm 0.2^{\P,\|}$
	40	$38.1 \pm 3.8^{*}$	$34.2 \pm 2.3^{\ddagger}$	$23.4 \pm 3.8^{\P}$
	80	$230.7 \pm 21.4^{\dagger}$	$120.5 \pm 16.9^{\S}$	$74.7 \pm 6.8^{\|}$
M-MØ	0	0.9 ± 0.1	3.1 ± 1.2	13.9 ± 2.2
	40	0.9 ± 0.1	2.6 ± 1.3	12.2 ± 3.1
	80	11.8 ± 0.3	17.5 ± 2.1	12.2 ± 2.9

$^{*}p < 0.005$; $^{\dagger}p < 0.005$; $^{\ddagger}p < 0.01$; $^{\S}p < 0.01$; $^{\P}p < 0.01$; $^{\|}p < 0.005$.

[a] $CD4^+$ T cells (1×10^5/well) were stimulated for 4 days with autologous GM-MØ or M-MØ at the indicated dose of macrophage. GM-MØ or M-MØ were pulsed with *M. leprae* and IFN-γ (100 IU/ml) on day 3, treated with CD40L (1 μg/ml) on day 4, and were used as APC on day 5. Representatives of three separate experiments are shown. Assays were done in triplicate, and results are expressed as mean ± SD. Groups with identical symbols were compared using Student's *t*-test.

Table 2
Contribution of IFN-γ and CD40L on T cell-stimulating activity of GM-MØ[a]

M. Leprae infection of GM-MØ (MOI: 80)	Treatment of *M. leprae*-infected GM-MØ with		IFN-γ (pg/ml) production by $CD4^+$ T cells after stimulation with GM-MØ at ratio (T:MØ)		
	IFN-γ (100 IU/ml)	CD40L (1.0 μg/ml)	2:1	4:1	8:1
−	+	+	2.3 ± 0.3	0.1 ± 0.2	0.8 ± 0.5
+	−	−	4.0 ± 1.1*	$5.5 \pm 1.9^{\S}$	$6.0 \pm 2.1^{\#}$
+	−	+	$21.4 \pm 3.1^{\dagger}$	$22.7 \pm 4.0^{\P}$	14.8 ± 2.2**
+	+	−	$20.3 \pm 1.7^{\ddagger}$	$15.9 \pm 1.3^{\|}$	$10.7 \pm 2.3^{\dagger\dagger}$
+	+	+	$226.1 \pm 20.9^{*\dagger\ddagger}$	$107.8 \pm 13.7^{\S\P\|}$	$94.8 \pm 9.7^{\#**\dagger\dagger}$

*$p < 0.005$; $^{\dagger}p < 0.005$; $^{\ddagger}p < 0.005$; $^{\S}p < 0.005$; $^{\P}p < 0.005$; $^{\|}p < 0.01$; $^{\#}p < 0.005$; **$p < 0.005$; $^{\dagger\dagger}p < 0.005$.

[a] $CD4^+$ T cells (1×10^5/well) were stimulated for 4 days with autologous GM-MØ at the indicated dose of macrophage. GM-MØ were pulsed with *M. leprae* and IFN-γ (100 IU/ml) on day 3, treated with CD40L (1 μg/ml) on day 4, and were used as APC on day 5. Representatives of three separate experiments are shown. Assays were done in triplicate, and the results are expressed as the mean ± SD. Groups with identical symbols were compared using Student's *t*-test.

MMP-II was observed on M-MØ, but, on GM-MØ, significant expression of MMP-II was induced. The expression was dependent on the dose of *M. leprae* (Fig. 5). However, the MMP-II expression on *M. leprae*-infected GM-MØ required both IFN-γ and CD40L, and apparent expression was not induced by sole treatment of macrophages with either IFN-γ or CD40L (data not shown).

4. Discussion

In order to avoid the intracellular multiplication and intercellular spread of *M. leprae*, the activation of adaptive immunity, especially of IFN-γ-producing type 1 T cells, plays an important role [5,6]. In fact, paucibacillary (tuberculoid) leprosy patients activate $CD4^+$ T cells through DC, although the bacteria cannot be eliminated completely [8,28]. The *M. leprae*-infected DC digest the bacteria and express dominant antigenic molecules for the efficient IFN-γ production from T cells [9]. In contrast, multibacillary (lepromatous) leprosy patients retain a large number of *M. leprae* in their MØ, and concordantly induce reduced levels or completely lack the ability to effectively stimulate T cells [11,12]. Since tissue resident MØ are heterogenous with regard to functional aspects [17,29], we assessed two different types of MØ: GM-MØ and M-MØ, and found that GM-MØ, but not M-MØ, stimulated T cells. GM-MØ were generated from monocytes using cytokine GM-CSF whilst M-MØ were produced using M-CSF. Although there were some differences in the expression levels of MHC class I, II, CD14 and CD209 Ags on GM-MØ and M-MØ, both forms were equally susceptible to mycobacteria as far as phagocytosis of BCG-GFP was examined. However, there was a striking difference between *M. leprae*-infected GM-MØ and M-MØ in the expression of antigenic molecules; only GM-MØ expressed MMP-II, which is one of the dominant antigenic molecules capable of stimulating T cells in *M. leprae*-infected individuals. The induction of MMP-II expression on GM-MØ requires not only GM-CSF, but also the co-stimulation of MØ with IFN-γ and CD40L. In case of *M. leprae*-infected DC, the phagosomal bacteria could be processed by lysosomal enzymes, and MMP-II expression was observed on DC [9]. The MMP-II expression observed on GM-MØ may indicate that at least some intracellular *M. leprae* were processed. However, the processing of *M. leprae* by GM-MØ still seemed partial, since the heat-killed *M. leprae* induced T cell activation more vigorously than live bacteria, and *M. leprae*-infected DC stimulated T cells more efficiently than GM-MØ, although other factors, such as an induction of IL-12, cannot be ruled out completely. The cell wall architecture including surface-exposed molecules, of heat-killed mycobacteria is globally altered [29,30], resulting in the exudation of some soluble antigenic molecules which may be feasibly digested in macrophages (unpublished observation). Therefore, T cells are more efficiently activated by heat-killed bacteria than by live bacteria.

Table 3
Comparison of T cell-stimulating activity of live and heat-inactivated *M. leprae*[a]

M. leprae pulsed on GM-MØ (MOI)	IFN-γ (pg/ml) production by $CD4^+$ T cells after stimulation with GM-MØ at ratio (T:MØ)		
	2:1	4:1	8:1
None	2.3 ± 1.1	2.1 ± 1.2	2.4 ± 0.9
HK (40)	406.5 ± 49.3*	$157.3 \pm 20.1^{\ddagger}$	$75.4 \pm 6.8^{\P}$
HK (80)	$399.8 \pm 33.2^{\dagger}$	$187.7 \pm 17.8^{\S}$	$106.9 \pm 11.2^{\|}$
Live (40)	101.5 ± 8.8*	$30.2 \pm 4.6^{\ddagger}$	$3.2 \pm 1.9^{\P}$
Live (80)	$152.0 \pm 12.7^{\dagger}$	$82.9 \pm 7.4^{\S}$	$32.7 \pm 2.8^{\|}$

*$p < 0.01$; $^{\dagger}p < 0.005$; $^{\ddagger}p < 0.005$; $^{\S}p < 0.005$; $^{\P}p < 0.005$; $^{\|}p < 0.005$.

[a] $CD4^+$ T cells (1×10^5/well) were stimulated for 4 days with autologous GM-MØ at the indicated dose of macrophage. GM-MØ were pulsed with either heat-killed (HK) or live *M. leprae* and IFN-γ (100 IU/ml) on day 3, treated with CD40L (1 μg/ml) on day 4, and were used as APC on day 5. Representatives of three separate experiments are shown. Assays were done in triplicate, and the results are expressed as the mean ± SD. Groups with identical symbols were compared using Student's *t*-test.

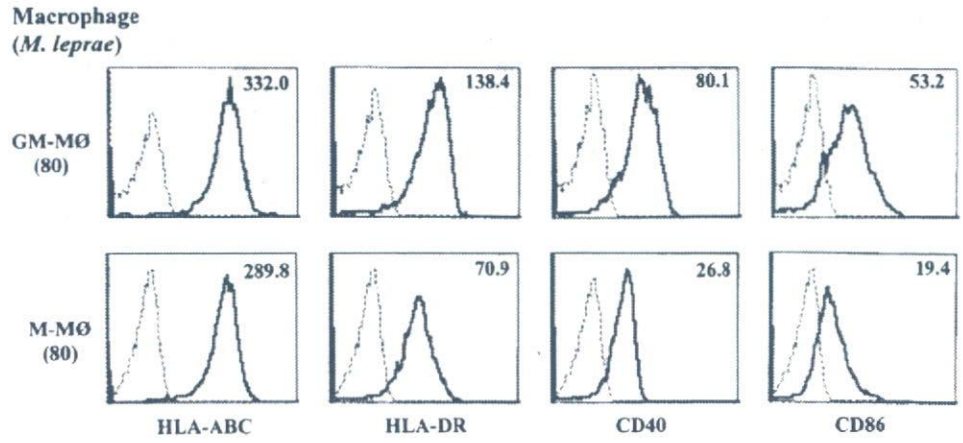

Fig. 3. Phenotype of *M. leprae*-infected GM-MØ and M-MØ. GM-MØ and M-MØ differentiated from monocytes by 3 days culture with rGM-CSF or rM-CSF were infected with *M. leprae*, treated with IFN-γ (100 IU/ml) on day 3, and further treated with CD40L (1 μg/ml) on day 4 of culture. On day 5, the phenotype of GM-MØ and M-MØ was analyzed. Dashed lines, isotype-matched control IgG; solid lines, mAb staining. The number represents the difference in mean fluorescence intensity between dashed and solid lines. Representatives of three independent experiments are shown.

Ottenhoff et al. have also reported that GM-CSF up-regulates the T cell-stimulating activity of MØ, but not M-CSF, and mycobacteria-infected GM-MØ promoted the type 1 cell-mediated immunity against pathogens [31]. Our observations are in line with their data and provide ways to enhance the cell-mediated immunity, especially in cases progressing towards lepromatous leprosy. To facilitate the T cell activation and MMP-II expression, it was required to use

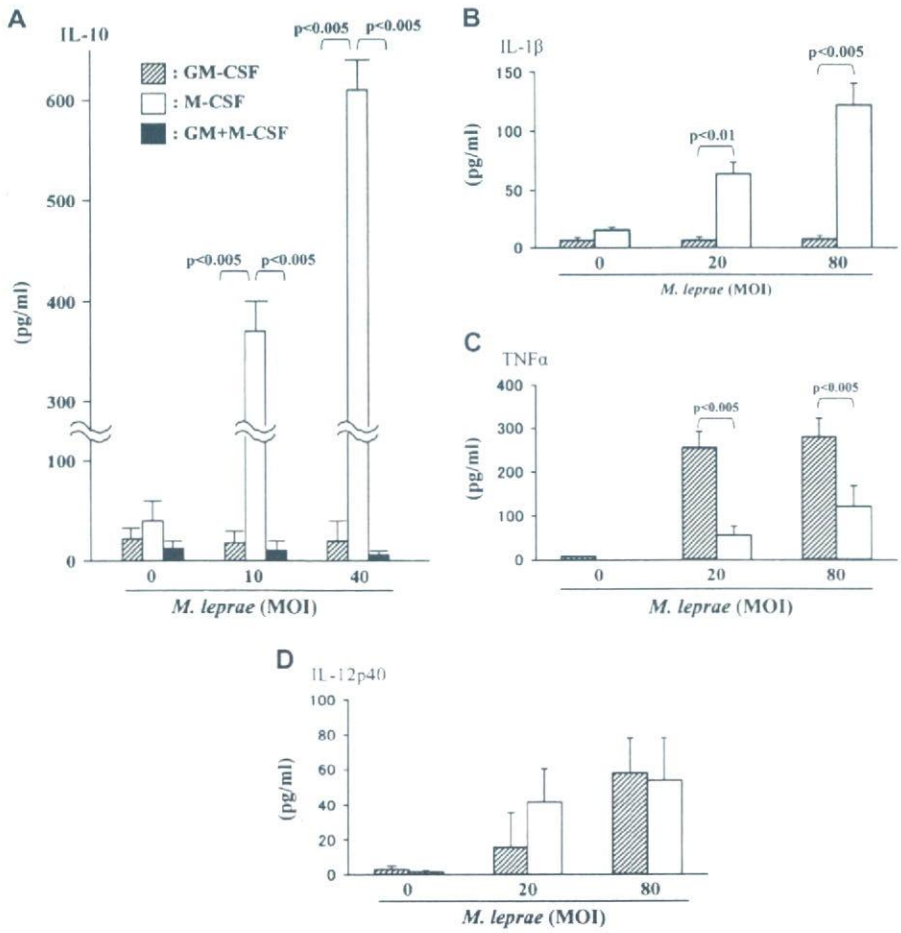

Fig. 4. Cytokine production from GM-MØ and M-MØ. MØ were differentiated by 3 days culture with rGM-CSF, rM-CSF or rGM-CSF + rM-CSF, and were stimulated with *M. leprae* for 24 h. The cytokines: (a) IL-10; (b) IL-1β; (c) TNFα; and (d) IL-12p40 were measured by ELISA. Representatives of three independent experiments are shown.